Segregation of Protein Synthesis Between the Cytoplasm
and Endoplasmic Reticulum of Eukaryotic Cells

by

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Dissertation submitted in partial fulfillment of
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ABSTRACT

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Abstract

The partitioning of translation to the outer membrane of the endoplasmic reticulum is a problem that has been the subject of inquiry since the discovery of the ribosome. The large degree to which ribosomes were found to be tethered to the membrane led to intense investigation of a series of related questions regarding the identity of those mRNAs that are translated on the endoplasmic reticulum, and the functions of that localization in cell stress. In this dissertation, I approach each of these questions in turn and work to reconcile my observations with those models that have been previously proposed. A theme of this work is the application of modern methods, particularly deep sequencing technology, to address problems that had largely been considered solved. The most prominently featured method is ribosome profiling, which is paired with classical biochemical and cell biological techniques. I arrive at several conclusions: 1) a significant fraction of all mRNAs is well represented on the endoplasmic reticulum membrane, 2) the properties of translation diverge substantially between membrane-associated and free ribosomes, and 3) the compartmentalization of translation can serve as an important variable in cell stress.

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1. Introduction: Translation and the endoplasmic reticulum

1.1 Beginnings: Palade and the electron microscope

The study of protein synthesis and the endoplasmic reticulum (ER) have been intertwined since the pioneering electron microscopy studies in the 1950s by George Palade. These works made two observations that took on enormous importance for the following 50 years. First, these studies were the first to identify the ER: a network of tubules within the cell separated from the cytosol by a membrane. Second, Palade and Claude noted that the outside of the ER membrane was dotted with points of high electron density – initially labelled simply as a "small particulate component of the cytoplasm" (Figure 1) (Palade, 1955). On further investigation, these points were found to be composed of RNA, and were named ribosomes (Palade, 1958). Pulse/chase experiments with radioactive amino acids found that the point of incorporation of free amino acids into protein was at the site of these ribosomes, which beforehand had no clear function. Together, these points initiated the long story of the ER, ribosomes, and the mechanisms by and purposes for which they associate.

The first answer to the question of purpose came quite early. Following closely behind the identification of the ribosome as the site of protein synthesis, Palade followed the fate of proteins that were new made in the endoplasmic reticulum (Caro and Palade, 1964; Jamieson and Palade, 1967a; Jamieson and Palade, 1967b; Jamieson and Palade, 1968a; Jamieson and Palade, 1968b; Jamieson and Palade, 1971). Palade again pulsed

cells with radioactive amino acids, the chased with cold amino acids for several time points. Afterwards, he used autoradiography and electron microscopy to track the localization of each of newly synthesized proteins. These landmark studies found that many newly synthesized proteins were initially present in the ER. As the chase time was extended, proteins were found in the early Golgi apparatus, the late Golgi, and many sere ultimately secreted. These works thereby defined the secretory pathway, and were a large part of what earned Palade and Claude the 1974 Nobel Prize in Physiology or Medicine.

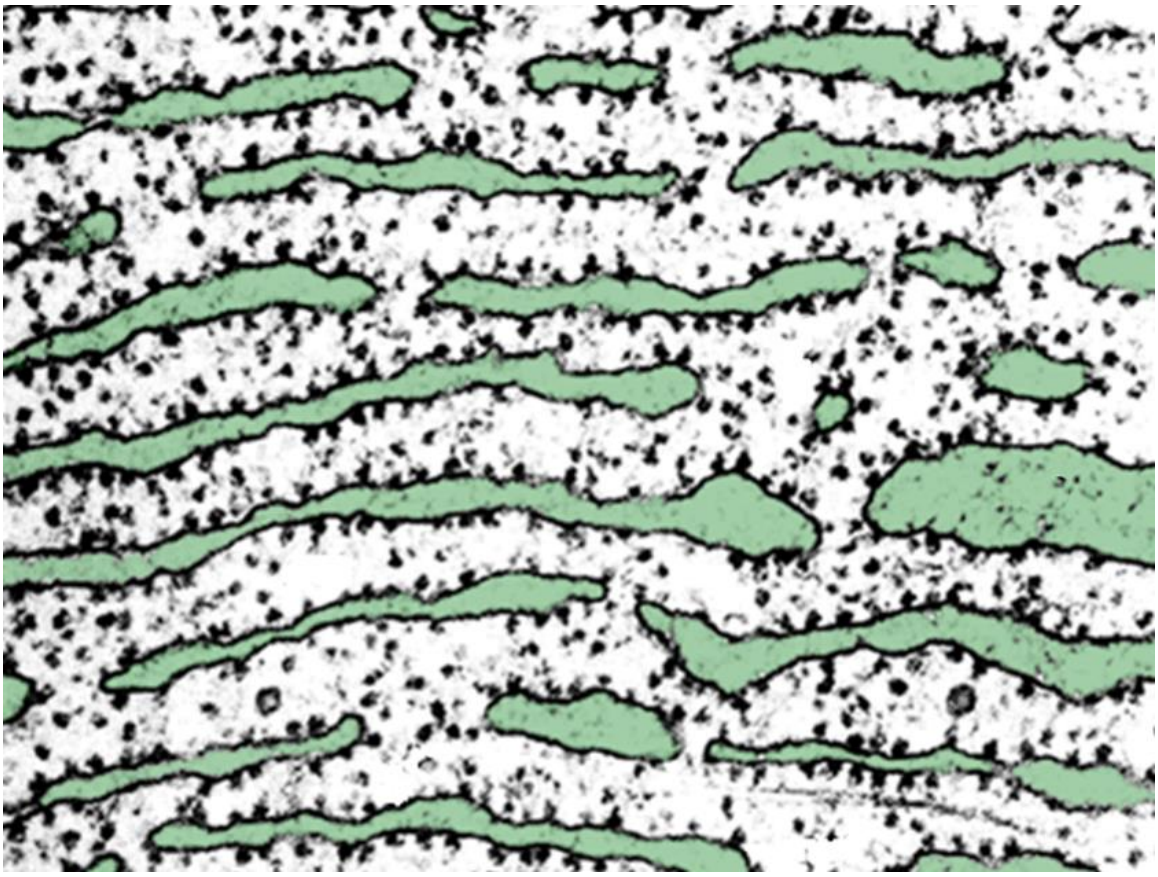


Figure 1: The ribosome-studded endoplasmic reticulum.

An electron microscopic image of a section of a pancreatic exocrine cell from (Palade, 1975). The lumen of the ER, false colored in green, is encapsulated by a membrane that is studded with ribosomes.

One of the most important conclusions from these studies was that ribosomes bound to the ER and the point at which proteins enter the secretory pathway (Siekevitz and Palade, 1960). That is, ER-associated ribosomes synthesize secretory and membrane proteins. This conclusion has held up quite well over time, with only a few exceptions identified. An extension of this conclusion, however, subsequently crept into the scientific lexicon: that ER-associated ribosomes only synthesize secreted and membrane proteins, but not cytosolic proteins; cytosolic ribosomes are responsible for the synthesis of cytosolic proteins. This particular conclusion, however, was not addressed in Palade's studies or other following work. Indeed, it would have been impossible to distinguish whether cytosolic proteins were synthesized by ER-bound ribosomes by these methods; upon completion of synthesis, cytosolic proteins would appear largely diffuse in the cytosol regardless of their site of synthesis, while nascent proteins on the ER would be essentially indistinguishable regardless of their ultimate cellular localization. Because Palade was only able to analyze discrete time points following the radioactive amino acid pulse, he could only conclude that *some* of the ribosomes on the ER synthesized secretory proteins. Palade noted in his Nobel lecture: "...the results are - to some extent - ambiguous, since - as isolated - [free and ER-associated] polysome classes carry newly synthesized proteins irrespective of the latter's final destination" (Palade, 1975).

Nonetheless, the idea of strict matching of ribosome localization and protein localization found its way into textbooks and scientific literature, guiding the study of translational compartmentalization for decades.

1.2 The Signal Recognition Particle

While the scope of activity of ER-bound ribosomes was still unclear, the observation that ER-bound ribosomes synthesize secreted and membrane proteins was at this point quite well established. The field therefore shifted its focus to mechanistic studies of the system. Prominent questions included modes of ribosome binding to the ER and mechanisms of protein translocation across the ER membrane. These studies were carried out most prominently in the laboratory of Günter Blobel at Rockefeller University. Over the course of several decades, Blobel and his colleagues used an *in vitro* rough microsome system to identify and characterize a system for co-translational recruitment of nascent proteins (and their associated polysomes) to the ER by a hydrophobic amino-terminal protein sequence (Blobel and Dobberstein, 1975a; Blobel and Dobberstein, 1975b). In the seminal set of experiments, a reaction that included rough microsomes stripped of ribosomes, purified ribosomes, and the mRNA encoding the light chain of IgG were incubated together. This combination resulted in the association of ribosomes with the microsomes and translocation of the light chain across the membrane, where its amino-terminal extension – termed a signal sequence – was cleaved to yield the mature protein. A series of intricate biochemical experiments by

Peter Walter identified a ribonuleoprotein, the Signal Recognition Particle (SRP), as being essential for this targeting (Walter and Blobel, 1981a; Walter and Blobel, 1981b; Walter et al., 1981). This targeting did not occur when an mRNA encoding a cytosolic protein, globin, was used instead (Walter and Blobel, 1981b).

Together, these studies defined a much more concrete system for the segregation of translation: translation initiates on ribosomes that are free in the cytosol, the resulting polysomes targeted to the ER by the encoded nascent chain, and ribosomes are released upon termination (Figure 2). For mRNAs that encode cytosolic proteins, there is no interaction with the Signal Recognition Particle, and therefore no association with the ER.

Figure 2: An early model for protein translocation across the ER membrane.

In 1975, Blobel and colleagues proposed a model for how proteins – and mRNAs – may be trafficked to the ER (Blobel, 2000). This model involves initiation in the cytosol, co-translational targeting of the nascent protein chain to the ER, and release of the ribosome upon termination.

Although this model was consistent with experiments from the Blobel lab, many questions remained regarding how well this is replicated in cells. Firstly, ribosomes do

not exchange between free and bound populations in a manner coupled with a single round of protein synthesis (Borgese et al., 1973), making the transient, nascent chain-dependent mechanism of ER:ribosome association unlikely to be the sole contributor to polysome recruitment to the ER. Secondly, the mRNA encoding the cytosolic Globin protein was detected in association with the ER in a proportion similar to the distribution of ribosomes, raising the possibility that ER-associated ribosomes take part in the synthesis of a wide range of proteins rather than just membrane and secretory proteins (Brennessel and Goldstein, 1975). This would require a mechanism for polysome interaction with the ER independent of the nascent protein. Thirdly, mRNAs were abundantly present on the ER after the pharmacological inhibition of translation, indicating that mRNAs themselves may have an independent means to association with the ER (Cardelli et al., 1976). Finally, and perhaps most simply, many polysomes are attached to the ER but have several of their component ribosomes a great distance from the ER, making it unlikely that they could participate in the synthesis of secretory proteins (Lee et al., 1971). As summarized by Palade: "To understand the situation, we need more information than we have at present on the relationship between free and attached ribosomes, on the position of polysomes at the time of initiation, and on the duration of polysomes attachment to the ER membrane" (Palade, 1975). It was apparent the story of translational compartmentalization was complex, but between the focus on the more straightforward study of signal sequence-mediated localization and lack of

obvious tools to further study the system, progress largely stagnated for the decades following the initial flurry of studies.

1.3 Re-opening the question of compartmentalization

During this period of relatively slow progress, a pair of papers developed hybridization techniques that began to hint at the actual complexity of translational compartmentalization (Mechler and Rabbitts, 1981; Mueckler and Pitot, 1982). In these experiments, mRNAs from the cytosol ($mRNA_{free}$) and ER ($mRNA_{bound}$) were separated. cDNAs were synthesized from each pool of mRNAs, then hybridized with the mRNAs of the other fraction. By assessing the degree to which each population hybridized with the other, these investigators could make conclusions regarding the complexity of each population and the differences between the two. Their conclusions stood in striking contrast to the developing consensus: instead of strict compartmentalization, these investigators found that most mRNAs were distributed between free and ER-bound ribosomes. Mueckler states: "[These results] demonstrate that various proteins are synthesized on both free and membrane-bound polysomes but preferentially on one or the other. In many cases the lack of an exclusive site of synthesis has been ignored or attributed to cross-contamination without presenting appropriate data. In light of the results presented here a re-evaluation of such data seems appropriate" (Mueckler and Pitot, 1982). However, this re-evaluation would not come for some time.

The question of how translation and mRNAs are distributed between free and ER-bound was incidentally re-opened in a 2000 study where Pat Brown's lab at Stanford University worked to identify new membrane proteins by taking advantage of their recently-developed cDNA microarray technology (Diehn et al., 2000). Here, Diehn and colleagues took a commonly accepted idea – that mRNAs encoding membrane and secreted proteins are the only mRNAs translated on the ER – and applied it by purified ER-associated and cytosolic mRNAs, then comparing each mRNA's relative distribution by microarray. In this analysis, one would hypothesize an obvious divergence between mRNAs encoding membrane and secreted proteins, which should be exclusive to the ER, and mRNAs encoding cytosolic proteins, which should be exclusive to the cytosol. Instead, Diehn found that the distributions of these two populations of mRNAs were largely overlapping, with virtually all mRNAs represented in the cytosol and ER. Although this study and a follow-up (Diehn et al., 2006) did not specifically address this overlap, it set the stage for other investigators to begin re-assessing the role of ER-bound ribosomes in protein synthesis.

Around the same time, the Nicchitta lab found that ER-associated ribosomes could participate in the initiation of translation – including initiation of translation of mRNAs encoding cytosolic proteins (Potter and Nicchitta, 2000). This was followed by a thorough documentation of mRNAs encoding cytosolic proteins being translated on the ER (Lerner et al., 2003) and an in-depth biochemical assessment of the divergent

properties of ER-bound and cytosolic ribosomes (Stephens and Nicchitta, 2008). Together, these studies began to ask a question that had sparsely been addressed since Palade was active: what are the roles of cytosolic and ER-bound ribosomes?

1.4 A framework for approaching translational localization

With several basic observations pointing towards a complex (Lerner et al., 2003; Mueckler and Pitot, 1982) and dynamic (Lerner and Nicchitta, 2006; Stephens et al., 2005) role for translational localization to the ER, I and other members of the Nicchitta lab set out to do a basic accounting of how translation is compartmentalized. To what degree are different mRNAs associated with the ER? How is their translation distributed between the cytosol and ER? How do the biochemical properties of translation differ in each compartment? To address these questions, I will work within a highly simplified conceptual framework (Figure 3). Here, ribosomes and each species of mRNA can have avidity for the ER. In either compartment, these mRNAs and ribosomes can be recruited into polyribosomes, which are generally the form that active ribosomes take. Alternatively, ribosomes may initiate translation of mRNAs in the cytosol, then be recruited to the ER, as would be predicted in the Signal Recognition Particle pathway. Although the actual situation in the cell is certainly more complex, this framework requires few assumptions and can provide an important, open-ended way to approach the question of translational localization.

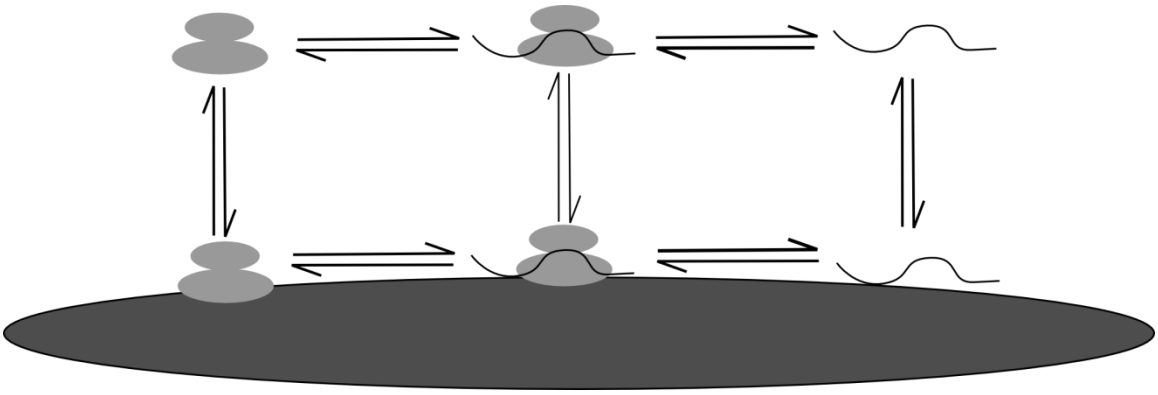


Figure 3: A conceptual model for considering translational compartmentalization

In this model, ribosomes and mRNAs may each associate with the ER (in grey) according to a particular avidity. In each compartment, mRNAs and ribosomes have a particular tendency to form into polyribosomes.

New technologies allow us to address these questions in ways that Palade could not have. Throughout this dissertation, I will take advantage of microarray and deep sequencing technology, which has dramatically expanded the experimental toolkit available to the RNA community. In particular, ribosome profiling (Ingolia et al., 2009) allows for transcriptome-scale analysis of translation, where mRNA partitioning and translation can be analyzed concurrently. In this technique, polyribosomes are digested with nuclease, then the ribosome-protected mRNA fragments are deeply sequenced.

This dissertation will discuss a series of studies that leverage these new technologies to provide new insights into the compartmentalization of the molecules that participate in protein synthesis, how these molecules interact in each compartment in order to give rise to productive protein synthesis, and how cells leverage this compartmentalization in order to enhance fitness.

2. Hierarchical organization of mRNA association with the ER

The work in this chapter introduces two general concepts. First, we address mRNA partitioning between the cytosol and ER not as a binary choice, but rather as a relative avidity that each mRNA possesses. The analysis of previously-published transcriptome-scale datasets through this lens reveal distinct behaviors of various classes of mRNAs. Further, we begin to define the biochemical mechanisms by which various mRNAs may associate with the ER: some classes of mRNA are dependent on the ribosome of ER association, while others are not.

Parts of this chapter also appear in the following: Chen Q, Jagannathan S, **Reid DW**, Zheng T, Nicchitta CV. (2011) Hierarchical regulation of mRNA localization to the endoplasmic reticulum. *Mol Biol Cell.* 22(14):2646-58. *The biochemical work in the chapter was performed primarily by Qiang Chen.*

2.1 Genome-scale compartmentalization of mRNAs to the ER

To assess the enrichment of a broad array of mRNAs on the ER, publicly available genome-scale subcellular mRNA partitioning data were analyzed using the cytosolic/nucleoplasmic/secretory cargo/resident endomembrane protein distribution model described in the preceding section. In this analysis, the mRNA subcellular distribution data set from K-562 leukemia cells, described in (Diehn et al., 2006), was examined. As shown in Figure 4A, peak K-562 gene enrichment was centered at $\log_2$ –

1.6, with maximal cytosol and ER enrichments extending from $\log_2$ –2.6 to 4.5, respectively. As in the mRNA distributions determined in J558 cells, the overall pattern of subcellular mRNA partitioning in K-562 cells is notable for the broad distribution of mRNAs between the cytosol and ER compartments, as well the differences in the maximal degree of enrichment in the ER relative to the cytosol (Figure 4A). The distribution pattern of mRNAs encoding cytosolic/nucleoplasmic proteins is shown in Figure 4B. This gene set was identified by selecting genes via the gene ontology (GO) category "cytoplasm" (GO: 0005737) and computationally filtered to remove any topogenic domain-encoding mRNAs that were included through inaccurate GO annotation. The K-562 cytoplasm cohort (1814 genes) displayed a peak at −1.8 $\log_2$(membrane/cytosol). A sizable fraction of mRNAs encoding cytosolic proteins was represented in the ER mRNA pool, with a small subset of the cytosolic protein-encoding mRNAs displaying relative ER enrichment levels that approximate those seen with topogenic domain-encoding mRNAs. The latter cohort was distinguished by its enrichment in mRNAs encoding proteins with a nuclear function as well as proteins functioning in cytoskeletal organization. To identify genes encoding resident proteins of the endomembrane system, a custom GO category, "endomembrane system," was defined to include proteins that reside in the rough ER and/or rough ER lumen (defined as bearing a C-terminal ER retention/retrieval motif), or are resident membrane proteins of the Golgi apparatus or lysosomes. This cohort (245 genes) displayed a peak

$\log_2$ enrichment of ~3.4, and ranged from −1.8 to 4.5 (Figure 4D). The subcohort of mRNAs displaying the lowest ER partitioning values was highly enriched in genes encoding enzymes functioning in lipid biosynthesis. In contrast, the subcohort displaying the highest ER partitioning values was enriched in genes encoding resident molecular chaperones of the ER lumen (i.e., GRP94 and calreticulin), proteins functioning in protein translocation/modification (β-subunit of the SRP receptor, ribophorin I), resident Golgi proteins functioning in oligosaccharide modification, and resident lysosomal proteins. Similar to the observations obtained in J558 cell fractionations, the genome-scale mRNA enrichment patterns of the cytosolic/nucleoplasmic and endomembrane system mRNA cohorts' genes displayed a bimodal distribution pattern with a clear bias to the ER compartment.

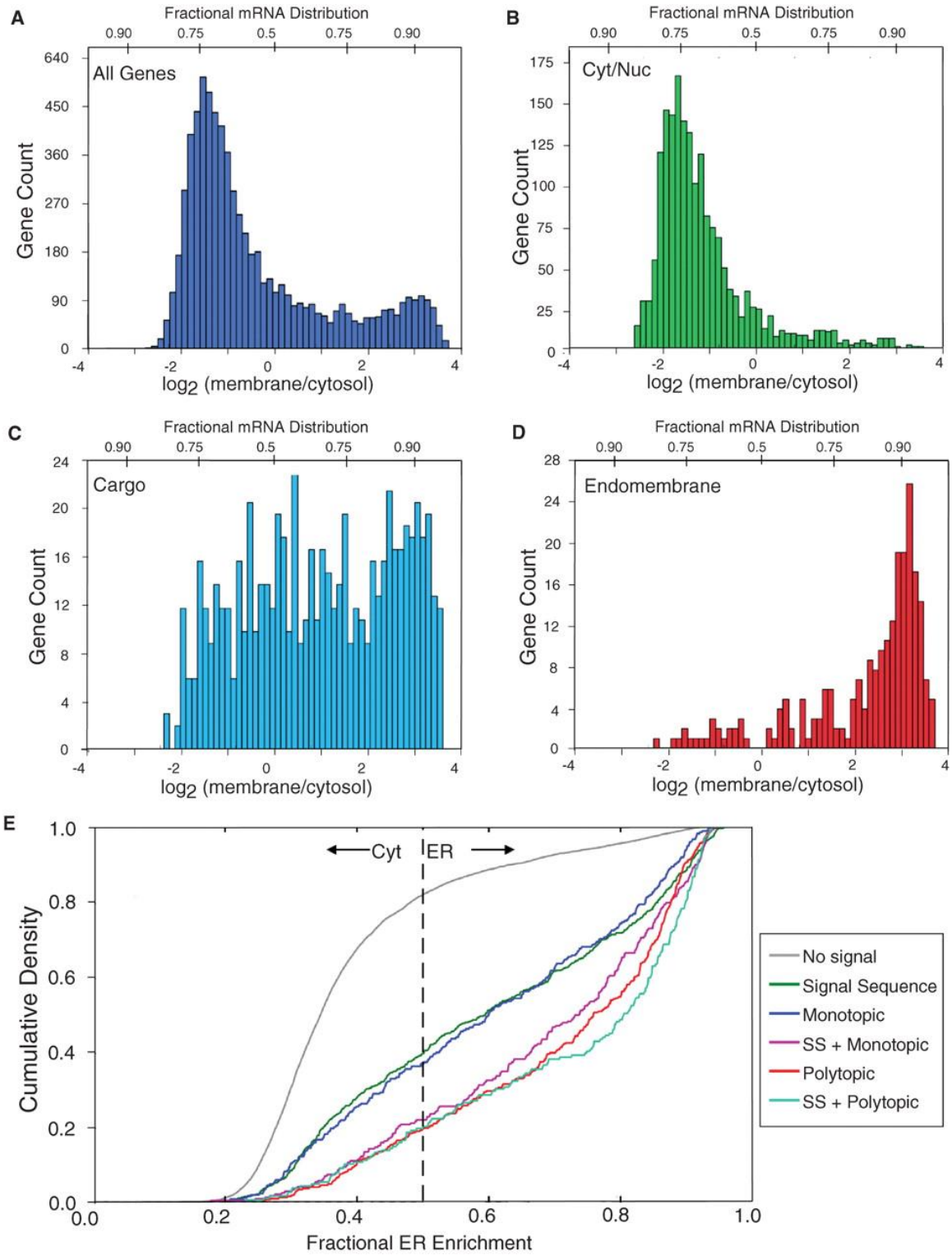

A
Fractional mRNA Distribution
0.90 0.75 0.5 0.75 0.90
All Genes
Gene Count
log2 (membrane/cytosol)
B
Cyt/Nuc
C
Cargo
D
Endomembrane
E
Cyt
ER
Cumulative Density
Fractional ER Enrichment
No signal
Signal Sequence
Monotopic
SS + Monotopic
Polytopic
SS + Polytopic

Figure 4: Genome-scale analysis of subcellular mRNA distribution reveals topogenic signal-independent partitioning patterns.

Publicly available subcellular gene production distribution data were analyzed by a three-cohort model. The genome database for the K-562 (human myelogenous leukemia) cell line was used. Gene product cohorts were identified by algorithmic sorting, using gene ontology (GO) criteria. (A) All genes. (B) Genes encoding cytosolic and nucleoplasmic proteins, selected via the GO category "cytoplasm" (GO: 0005737) and filtered to remove topogenic signal-encoding genes. (C) Secretory pathway cargo. The K-562 gene set was sorted using the GO categories "extracellular" (GO: 0005615) and "plasma membrane" (GO: 0005886). (D) Endomembrane system. The K-562 gene set was sorted using a custom GO category "endomembrane" to include genes whose translation products reside in the ER membrane, the ER lumen, or the Golgi apparatus or lysosomes. (E) Subcellular mRNA distributions were analyzed by cumulative density distribution, using a six-cohort model: no topogenic signal, signal sequence-encoding, single transmembrane domain-encoding (monotopic), multiple transmembrane domain-encoding (polytopic), and single/multi-transmembrane domain plus signal sequence.

As observed in the subgenomic analysis, the bimodal distribution pattern displayed by the K-562 cytosolic/nucleoplasmic and endomembrane system mRNA cohorts was compromised when mRNAs encoding secretory pathway cargo (668 genes) were included in the analysis (Figure 4C). This category was selected by filtering the K-562 gene set by the GO categories "extracellular" (GO:0005615) and "plasma membrane" (GO:0005886) and displayed $\log_2$ (membrane/cytosol) enrichment values ranging from −1.9 to 4.2. At the genome scale, no clear correlation between signal peptide H region length and subcellular distribution was discernible, with both the high and low relative ER membrane enrichment cohorts displaying strong signal peptide H region SignalP scores (Bendtsen et al., 2004). As observed in the qPCR array studies (Chen et al., 2011), the secretory pathway cargo-encoding mRNA cohort displayed a broad subcellular

distribution pattern, with a substantial number of topogenic domain-encoding mRNAs partitioning to the cytosol.

To further investigate the role of topogenic signals in the regulation of subcellular mRNA partitioning, $\log_2$ (membrane/cytosol) K-562 gene distributions were evaluated with respect to encoded topogenic signals (signal peptide, monotopic membrane protein, polytopic membrane protein). Using the programs SignalP and TMHMM, genes were classified into six categories: lacking topogenic signal, signal peptide-encoding, single transmembrane domain-encoding, multiple transmembrane domain-encoding, single transmembrane domain and signal peptide-encoding, and lastly, multiple transmembrane domain and signal peptide-encoding (Bendtsen et al., 2004; Krogh et al., 2001). Cumulative density functions were then generated for each cohort (Figure 4E). In the case of mRNAs lacking an encoded topogenic signal (i.e., cytosolic/nucleoplasmic), the gene density function is curvilinear, with a substantial gene representation at $\log_2 = 0$ and a cohort of genes displaying noncanonical enrichment in the ER. Plots depicting topogenic signal-encoding genes are also curvilinear and include a substantial fraction of gene products that are cross-represented in the cytosol and ER compartments. For those genes encoding mono- and polytopic transmembrane domain-bearing proteins, a more pronounced bias to ER enrichment was observed, suggesting that transmembrane domain topogenic signals are dominant to signal peptides (Figure 4E). Interestingly, overall ER-enrichment values were most enhanced

for those mRNAs encoding both transmembrane domains and signal peptides (Figure 4E). The extended fractional enrichments in the cumulative density plots suggest that multiple processes contribute to individual subcellular mRNA distribution patterns, a conclusion that is supported by the divergence between the observed cumulative density distributions and the distributions predicted by a positive selection mechanism of mRNA partitioning to the ER.

In summary, analysis of genome-scale subcellular mRNA partitioning data via the three-cohort model described above supports the view that mRNA partitioning is under hierarchical regulation and suggests that mRNAs encoding resident proteins of the endomembrane system contain localization information that is dominant to that expressed by topogenic protein-encoded signals. When sorted with respect to topogenic signals, the data also demonstrate that transmembrane domain topogenic signals are dominant to signal peptides in conferring an ER-enriched subcellular distribution pattern.

Given the substantial variations in the subcellular partitioning patterns of mRNA cohorts that share the property of an encoded topogenic domain (secretory pathway cargo and endomembrane system), we postulated that a hierarchical mechanism of mRNA partitioning to the ER would include cohort-specific modes of biochemical association with the ER membrane. This hypothesis was examined in the experiments described below.

2.2 mRNAs associate with the ER by distinct biochemical mechanisms

Expanding on the qPCR and cDNA microarray studies described above, where the endomembrane system-encoding mRNA cohort was distinguished from the secretory pathway cargo-encoding cohort via $\log_2$(membrane/cytosol) enrichment values, we sought to determine whether endomembrane system-encoding and secretory pathway cargo-encoding mRNAs displayed similar or distinct modes of interaction with the ER membrane. To this end, mRNA–ER membrane interactions were examined by biochemical fractionation of equilibrium density gradient–purified rough microsomes (RM) (Stephens et al., 2005; Stephens et al., 2008).

Past studies have established that exposure of RM to high salt concentrations can elicit 80S ribosome release from the ER membrane, whereas divalent cation chelators (e.g., EDTA) elicit ribosomal subunit dissociation and efficient 40S and partial 60S ribosomal subunit release from the ER membrane (Adelman et al., 1973; Sabatini et al., 1966). To assay for ribosome-dependent mRNA–ER interactions, RM were prepared from J558 plasmacytoma and H929 myeloma cells; adjusted to 0.5M KCl, 20 mM EDTA, or 0.5M KCl/20 mM EDTA; and subjected to ultracentrifugation to separate the membrane-associated and released fractions. The two fractions were assayed via analysis of 28S/18S rRNA for ribosome content and via Northern blot for mRNA. As shown in Figure 5A,B when RM suspensions were diluted into physiological salt solutions and subjected to ultracentrifugation, ribosomes and mRNAs encoding the

resident ER chaperones GRP94 and BiP (J558 cells), or GRP94 and calreticulin (H929 cells), were efficiently recovered in the membrane (P) fraction. Also recovered in the membrane fraction were mRNAs encoding the secretory pathway cargo proteins λ light chain (J558 cells) and κ light chain (H929 cells). Following dilution of the RM fractions with 0.5 M KCl solutions, a partial release of ribosomes was observed for both J558 and H929 RM, with modest effects on mRNA distributions. As noted in past studies, ribosomes released under such conditions include vacant 80S monosomes (Stephens et al., 2005). Under experimental conditions, where ribosomes were dissociated into their component subunits (addition of EDTA), the membrane association behavior of the two mRNA species could be clearly distinguished. In the presence of 20 mM EDTA, mRNAs encoding the secretory pathway cargo proteins λ and κ light chain were recovered in the supernatant (S) fraction, whereas mRNAs encoding the resident ER proteins GRP94, BiP, and calreticulin were recovered in the membrane fraction (Figure 5A,B). These differences were further distinguished by extraction of RM in high salt/EDTA-supplemented buffers. Under these conditions, mRNAs encoding ER-resident proteins remained tightly associated with the ER membrane, whereas secretory pathway-encoding proteins and ribosomes were efficiently released into the supernatant fraction. These data identify two biochemically distinguishable modes of mRNA association with the ER. In one mode, displayed by mRNAs encoding the secretory pathway cargo proteins λ and κ light chain, mRNAs displayed membrane-binding properties similar to

those established for ribosomes and were released upon addition of high salt/EDTA-containing buffers. In contrast, mRNAs encoding resident ER proteins remained predominantly membrane-associated under experimental conditions that elicited ribosome dissociation and release. These data suggest a model where mRNAs can be localized to the ER through distinct, though not necessarily exclusive, modes via their functional association with membrane-bound ribosomes and/or via salt and divalent cation-insensitive, ribosome-independent interactions with components of the ER membrane.

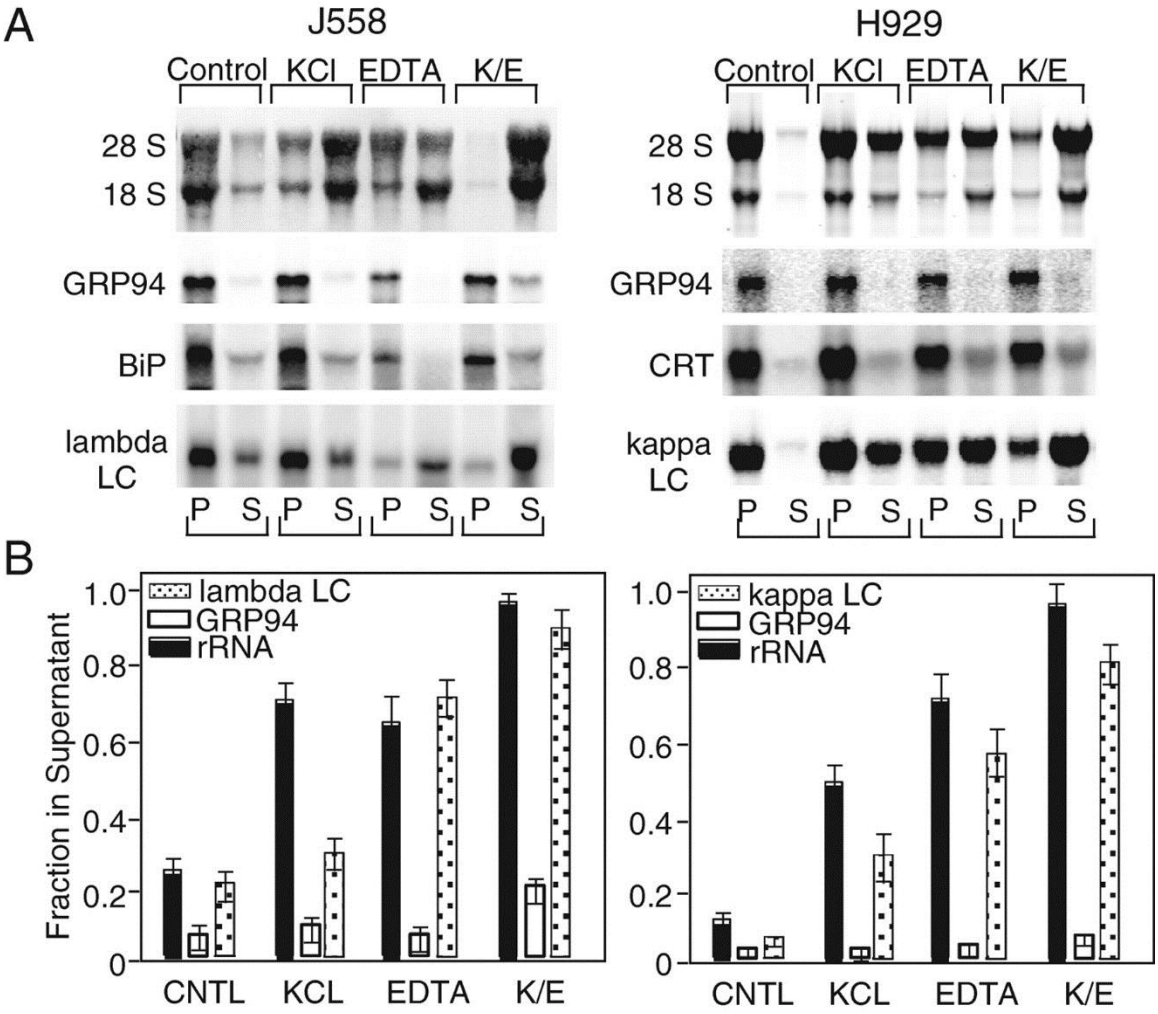


Figure 5: mRNAs display cohort-specific modes of interaction with the ER membrane.

RM were purified from J558 murine plasmacytoma and H929 human myeloma cells by equilibrium density gradient centrifugation and mRNA–ER membrane interactions were determined by biochemical fractionation. RM suspensions were diluted in physiological salts buffer (Control), 0.5 M KCl (KCl), 20 mM EDTA (EDTA), or 0.5 M KCl/20 mM EDTA (K/E) and incubated on ice. Membrane-bound (P) and released (S) fractions were separated by ultracentrifugation and total RNA was isolated. (A) rRNA (28S, 18S) distributions were determined by dye staining, and mRNAs encoding the ER-resident proteins GRP94 (J558, H929), BiP (J558), calreticulin (CRT)(H929), λ light chain (J558), and κ light (chain (H929) were determined by Northern blot analysis. (B) Digital images for rRNA and mRNA distributions are depicted. rRNA and mRNA distributions were quantified by ImageJ analysis of SYBR Safe stained RNA gels or phosphorimager scans

of Northern blots. Data represent mean ± SD of seven (J558) or three (H929) individual experiments.

As a further test of this model, an alternative biochemical method for disrupting peripheral protein–membrane interactions was examined. Previous studies have demonstrated that high concentrations of protonated amines (e.g., neutral Tris solution) efficiently extract the peripheral membrane protein fraction of coated vesicles (Keen et al., 1979). This experimental approach was adapted to the analysis of ribosome–mRNA interactions in experiments where RM were treated with increasing concentrations of protonated amines, and ER membrane–mRNA interactions were examined by the centrifugation assay detailed above. The results of these studies are depicted in Figure 6A,B. When J558 RM were treated with increasing concentrations of Tris/Cl (pKa = 8.3), a progressive release of ribosomes was observed (Figure 6A,B). Ribosome release was discernibly less efficient in the presence of increasing concentrations of imidazole/Cl (pKa = 5.9), an observation consistent with the requirement for the protonated form of the base in the disruption of peripheral membrane interactions (Keen et al., 1979). At Tris/Cl or imidazole/Cl concentrations up to 0.25 M, BiP- and λ light chain–encoding mRNAs were retained on the ER. However, in the presence of 0.5 M Tris/Cl, BiP-encoding mRNAs were retained on the ER, whereas λ light chain mRNAs were efficiently released.

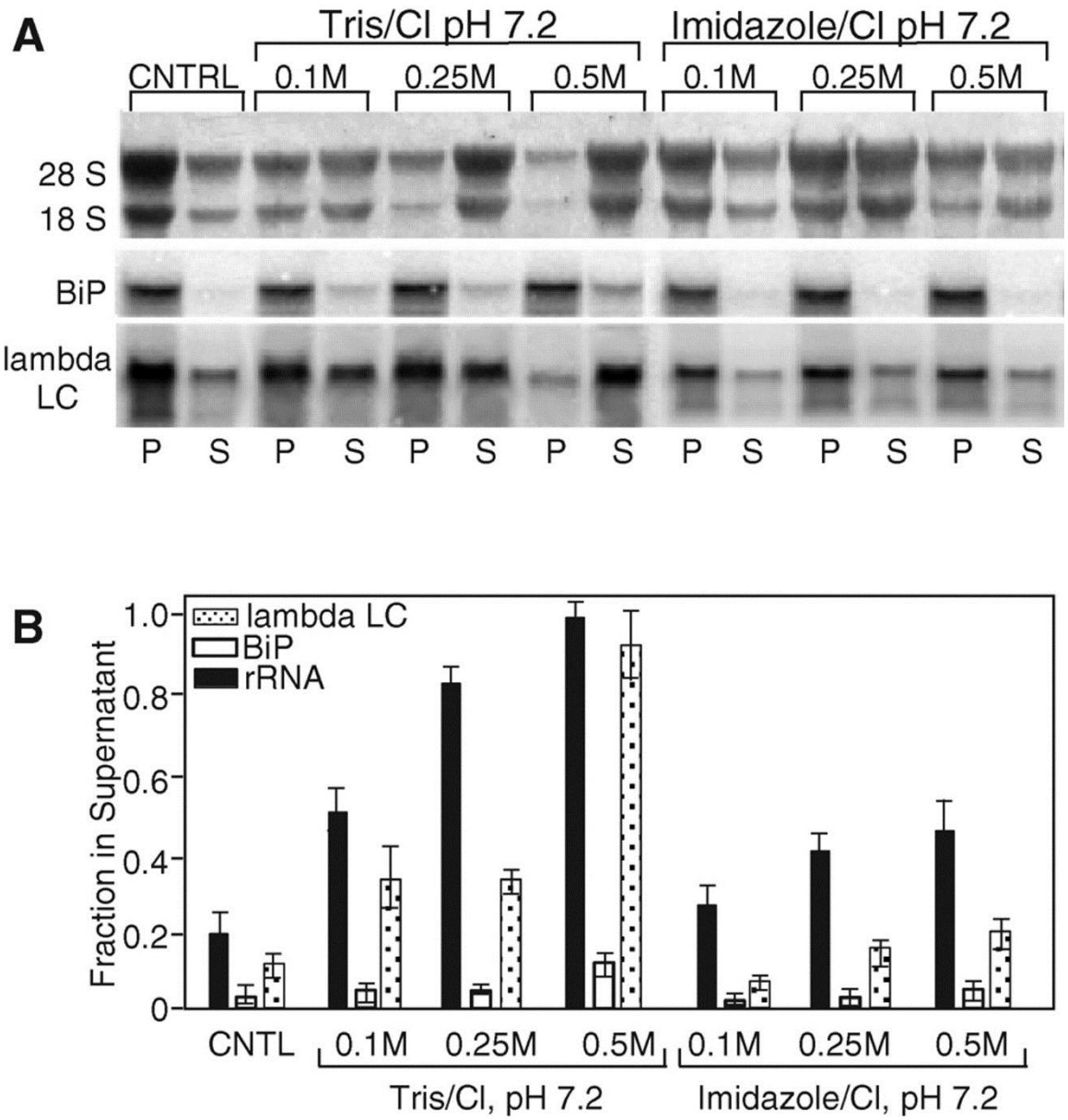


Figure 6: mRNA–ER binding interactions of mRNAs are distinguished by protonated amine extraction.

RM were purified from J558 murine plasmacytoma cells by equilibrium density gradient centrifugation and mRNA–ER membrane interactions were examined by extraction with the protonated amine buffers (neutral Tris or neutral imidazole). J558 RM suspensions were diluted in physiological salts buffer (Control), neutral Tris/HCl (0.1, 0.25, 0.5 M), or neutral imidazole/HCl (0.1, 0.25, 0.5 M), and incubated on ice, and the membrane-associated and -released fractions were isolated by ultracentrifugation. Samples were

processed as described in the Figure 4 caption. (A) Digital images of rRNA gels and phosphorimager data (BiP, λ light chain). (B) Data from three independent experiments are summarized, with mean ± SD values indicated.

2.3 The role of topogenic signals in directing mRNA localization to the ER

The SRP pathway provides a well-established, signal peptide- and translation-dependent mechanism functioning in mRNA localization to the ER (Walter and Blobel, 1980; Walter and Blobel, 1981b; Walter et al., 1981). Though it is generally accepted that the SRP pathway directs mRNA localization to the ER, past studies of mRNA partitioning between the cytosol and ER have indicated that multiple pathways likely function in mRNA localization to the ER (Diehn et al., 2000; Mueckler and Pitot, 1981; Mueckler and Pitot, 1982). We have examined subcellular mRNA distribution patterns, and consistent with past studies, we have also observed broadly overlapping distributions of mRNAs between the cytosol and ER compartments. Through analysis of the subcellular partitioning of defined mRNA cohorts, the studies described here now demonstrate that topogenic signals comprise one of likely many mRNA localization elements functioning in mRNA localization to the ER. Importantly, in such a model, topogenic signals are not uniquely predictive of an ER-enriched mRNA distribution. Topogenic signals are, however, essential for protein translocation across the ER membrane, and we propose that topogenic signal function in mRNA localization to the ER is distinct from that operating in protein translocation. This hypothesis is supported by findings in yeast and mammalian cells demonstrating that the SRP pathway is not

essential for viability. However, the loss of SRP pathway function can negatively impact the efficiency of protein translocation, and clearly presents a significant, though surmountable, physiological challenge to cells (Hann et al., 1989; Mutka and Walter, 2001). The observations reported here and in a past report (Pyhtila et al., 2008) provide some insight into why this might be. For example, those mRNAs for which ER localization can proceed in the absence of SRP necessarily would be insensitive to the loss of SRP function. Also, and as discussed further below, the observations that ER-bound ribosomes can initiate mRNA translation, and that secretory proteins whose synthesis is initiated on ER-bound ribosomes are translocated in the absence of SRP receptor function, suggest additional mechanisms whereby loss of SRP function can be tolerated (Potter and Nicchitta, 2000; Potter et al., 2001).

2.4 A broad spectrum of mRNAs on the ER

We observed that the cytosolic/nucleoplasmic protein-encoding mRNA pool was well represented on the ER, a finding that suggests a broad role for the ER in global protein synthesis. The mechanism or mechanisms enabling the translation of cytosolic/nucleoplasmic protein-encoding mRNAs on the ER remain to be determined. It is possible that this phenomenon reflects a stochastic distribution of mRNAs between free and membrane-bound ribosomes. For example, if newly exported mRNAs are accessible to translation by membrane-bound and cytosolic ribosomes alike, mRNAs that lack specific compartmental enrichment information would be predicted to

partition between both ribosome pools, with steady-state mRNA subcellular distribution profiles determined by both ribosome distribution and the elongation-coupled ribosome release pathway previously proposed (Potter and Nicchitta, 2000; Potter et al., 2001). Given the physical and functional continuity between the outer nuclear envelope and the ER, such a mechanism may favor an ER locale for the translation of newly exported mRNAs. In models where the initiation of translation is restricted to the cytoplasm, ER-bound ribosomes would necessarily need to be refractory to initiation. However, where this has been experimentally examined, it was observed that ER-bound ribosomes were capable of de novo initiation of mRNAs encoding cytosolic and secretory proteins alike (Nicchitta et al., 2005; Potter and Nicchitta, 2000; Potter et al., 2001). Regardless of the mechanisms governing the subcellular site of initiation, the fact that mRNAs encoding cytosolic/nucleoplasmic proteins are abundantly represented on ER-bound ribosomes indicates multiple pathways function to establish subcellular mRNA distributions.

In both subgenomic and genomic analyses, mRNAs encoding a subset of soluble protein kinases and transcription factors displayed noncanonical enrichments on the ER. The biological function or functions enabled through such noncanonical localization events remain to be determined. In all likelihood, such noncanonical localization patterns have direct biological functions, as proposed by Krause and colleagues (Lecuyer et al., 2009; Lecuyer et al., 2007). In one prominent mechanism, displayed by the stress-response transcription factors Hac1 (yeast) and XBP1 (higher eukaryotes), mRNA-intrinsic

localization information (Hac1) and/or nascent chain-encoded translational regulatory elements (XBP1) direct noncanonical localization to the ER as an essential regulatory step in the mRNA-processing reactions that yield the activation of transcription factor function (Aragon et al., 2009).

3. A primary role for ER-bound ribosomes in cellular protein synthesis

Having found that virtually all mRNAs are represented on the ER, we next sought to assess the properties of translation on the ER relative to the cytosol. Using biochemical and informatics methods, we find that 1) mRNAs are more densely loaded with ribosome on the ER than in the cytosol and 2) virtually all mRNAs, including those encoding cytoplasmic proteins, are translated on the ER to a large extent. This work identifies the ER as a primary cellular compartment for the synthesis of virtually all proteins.

Parts of this chapter also appear in the following: **Reid DW**, Nicchitta CV. (2012) Primary role for endoplasmic reticulum-bound ribosomes in cellular translation identified by ribosome profiling. *J Biol Chem*. 287(8):5518-27.

3.1 Biochemical assessment of translation in cellular compartments

To study mRNA distribution and translation on cytosolic and ER-bound polyribosome pools, HEK293 cells were fractionated using a previously described sequential detergent extraction protocol that generates highly enriched cytosolic and ER-derived fractions

(Jagannathan et al., 2011; Lerner et al., 2003; Stephens et al., 2008). To assess polyribosome structure, sucrose density gradient velocity sedimentation studies were performed for ER- and cytosol-derived fractions (Figure 7A,B). The cytosolic and ER-derived polyribosome profiles were similar, with abundant polyribosomes present in each subcellular fraction and the cytosol fraction containing somewhat higher levels of 40S, 60S ribosomal subunits and 80S ribosomes relative to the polysomal population. To quantify subcellular ribosome quantities and distributions, cell cultures were metabolically labeled to steady state with [^{3}H] uridine and the mRNA-associated ribosomes purified by native chromatography on oligo-dT (7) cellulose resin, then quantified by scintillation spectrometry (Figure 7C). Consistent with the polyribosome profiles illustrated in Figure 7A-B, similar quantities of mRNA-associated ribosomes were recovered from each fraction. To quantify total mRNA levels in the cytosol- and ER-derived fractions, total mRNA was selected from each fraction using subtractive hybridization against rRNAs and quantities determined by Bioanalyzer analysis. In contrast to ribosomal distributions, total mRNA levels differed in the two fractions, with total mRNA levels being approximately two fold higher in the cytosol (Figure 7D). These data suggest that, on average, ribosomes are more densely loaded on ER-bound vs. cytosolic mRNAs.

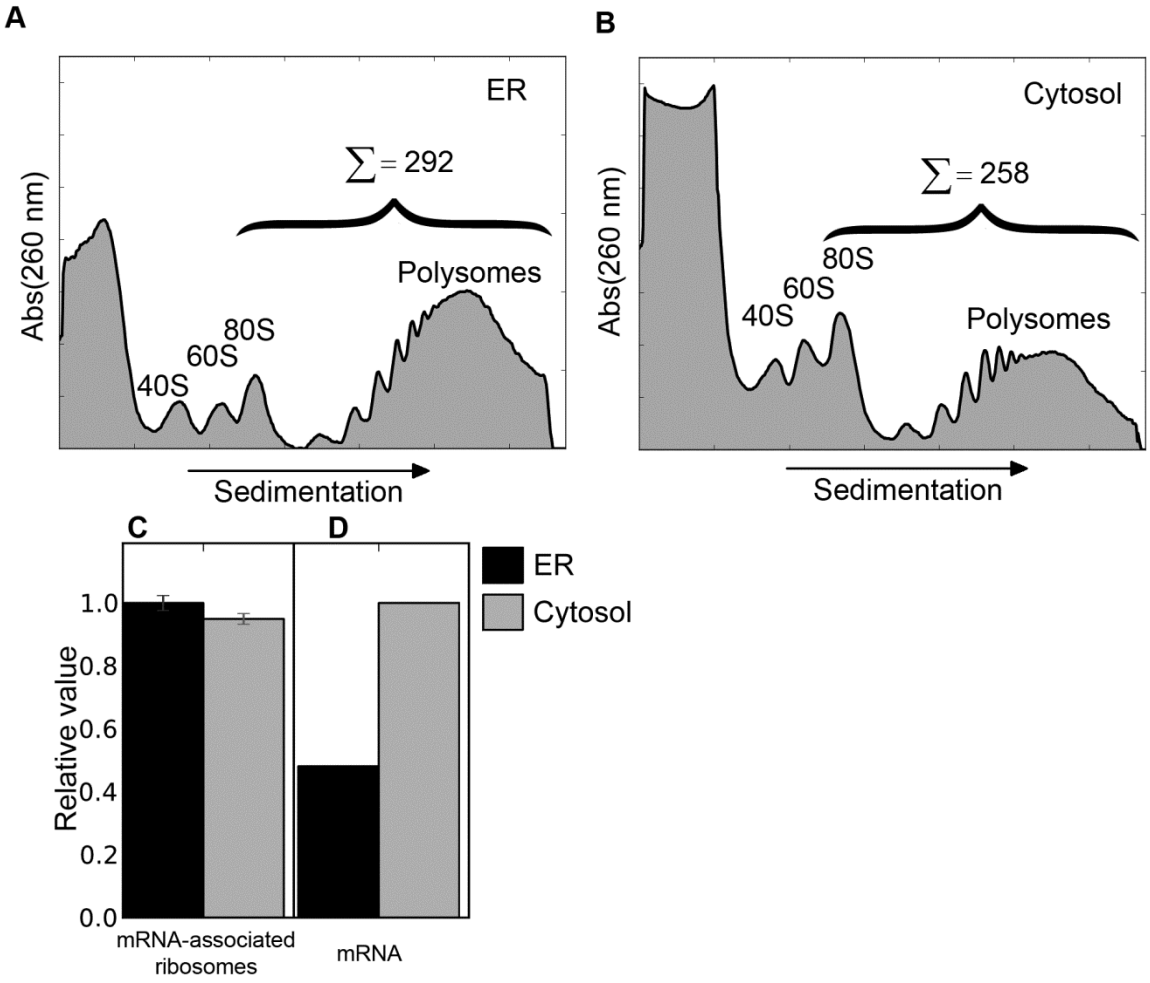


Figure 7: Biochemical characterization of translation in cytosol and ER.

A and *B*, subcellular fractions representing the cytosol (*A*) and ER (*B*) were analyzed by sucrose gradient velocity sedimentation. The peaks corresponding to the 40 S, 60 S, and 80 S ribosomes are indicated, as are the relative quantities of combined 80 S ribosomes and polyribosomes. *C*, the subcellular distribution of ribosome-associated mRNA was determined by native oligo(dT) affinity purification of [^{3}H]uridine-labeled polyribosomes and quantification of EDTA-releasable ribosomal subunits. Subcellular mRNA distributions were determined by treating total RNA fractions with RiboMinus, to remove rRNAs, and spectrophotometric quantification of mRNA levels. Values were normalized to a maximum value of 1. *Error bars* represent ± one S.D.

To evaluate the translational status of ribosomes in the ER and cytosol, cells were labeled with [^{35}S] Met/Cys for 2 min, then treated with puromycin, which elicits the premature termination of translation and partial polyribosome breakdown (Blobel and Sabatini, 1971; Rabinovitz and Fisher, 1962). Puromycin treatment elicited breakdown of polyribosomes in both the cytosol and ER, indicating that both populations of polyribosomes were translationally active (Figure 8). Corroborating this conclusion, [^{35}S] Met/Cys was incorporated into nascent polypeptides in each polyribosome population and abolished upon puromycin treatment. To confirm that mRNAs encoding cytosolic proteins were among the population of actively translating polysomes that were disassembled by puromycin on the ER, semi-quantitative PCR targeting ACTB and GAPDH, both of which lack topogenic signals, showed that each mRNA moved to a markedly lighter polysome fraction or lost polysome association altogether in both the cytosol and ER. GRP94, which encodes a topogenic signal, is similarly reduced in polysomes on the ER, indicating that each class of mRNA is actively translated on the ER. Combined with previous work indicating similar elongation rates for cytosolic and ER-bound ribosomes (Ingolia et al., 2011; Stephens and Nicchitta, 2008), these experiments support ribosome loading status as a reasonable proxy for translation in each cellular compartment.

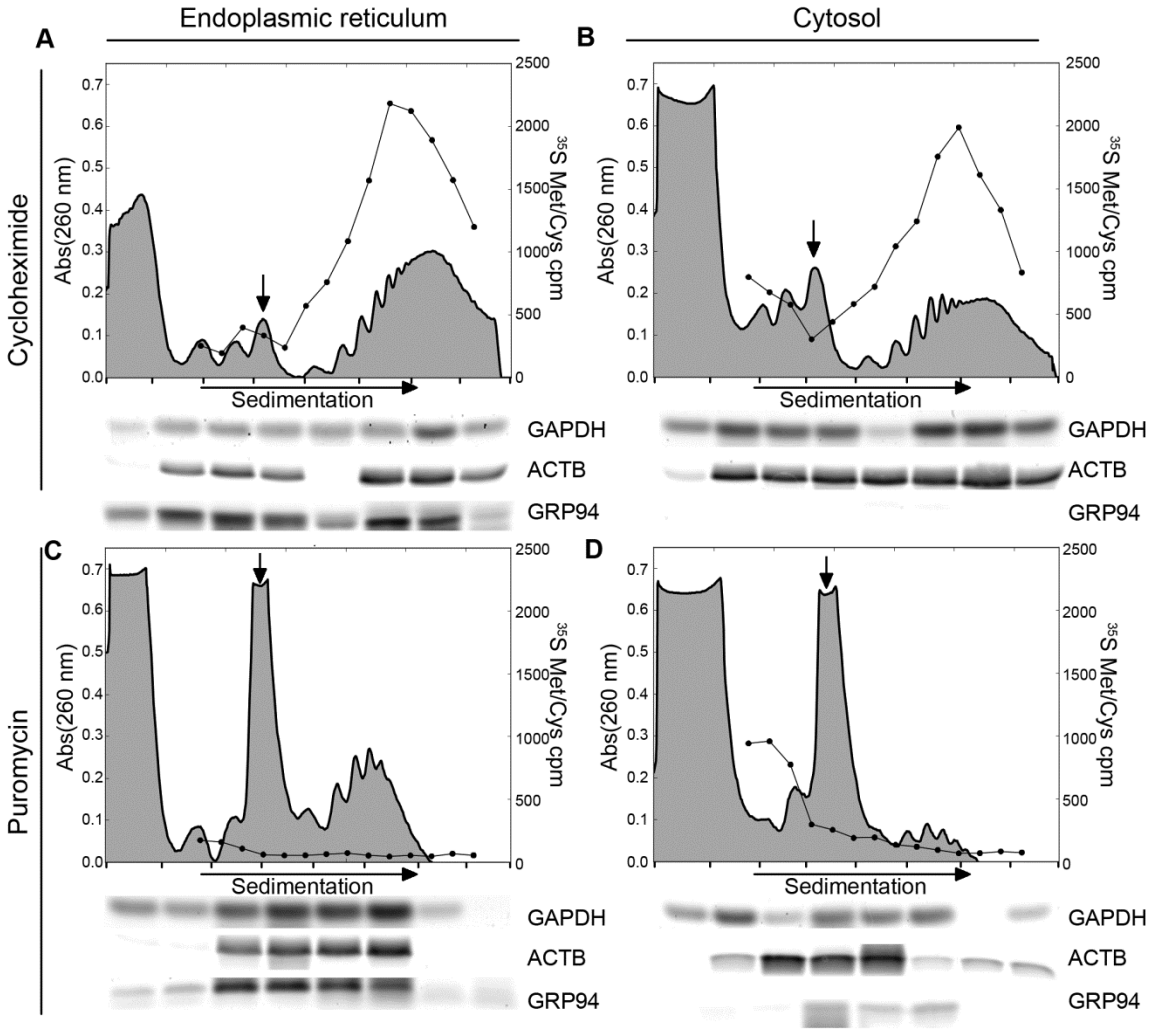


Figure 8: Analysis of compartmental mRNA translational status.

A–D, polyribosome profiles from the cytosol and ER in cycloheximide- (*A* and *B*) and puromycin- (*C* and *D*) treated cells are illustrated. The *downward-facing arrows* indicate the migration position of 80 S ribosomes. mRNA translational status was determined by [^{35}S]Cys/Met incorporation and is depicted in the line graphs. Total RNA was isolated from gradient fractions, and the relative levels of the indicated mRNAs were determined by semiquantitative PCR.

3.2 Ribosome footprinting analysis of ribosome loading in the cytosol and ER compartments of HEK293 cells.

To obtain a survey of subcellular mRNA ribosome loading status, we utilized ribosome footprinting coupled with deep sequencing. Here, cytosolic and ER-associated polyribosomes were digested with micrococcal nuclease (Arnone et al., 1971; Heins et al., 1967) to yield intact 80S ribosomes and their associated protected mRNA fragments (Wolin and Walter, 1988). The ribosome-protected mRNA fragment complexes were isolated by ultracentrifugation, subjected to phenol/chloroform extraction, and the ca. 35 nt nuclease-protected mRNA fragments purified by acrylamide gel electrophoresis (Figure 9). cDNA libraries were prepared using the SOLiD small RNA expression kit protocol and the library deeply sequenced on the SOLiD4 platform (Applied Biosystems). In parallel, total mRNA samples were prepared for deep sequencing so that the abundance of mRNAs in each compartment could be defined, allowing evaluation of ribosome footprints per mRNA, or ribosome density, for individual mRNAs.

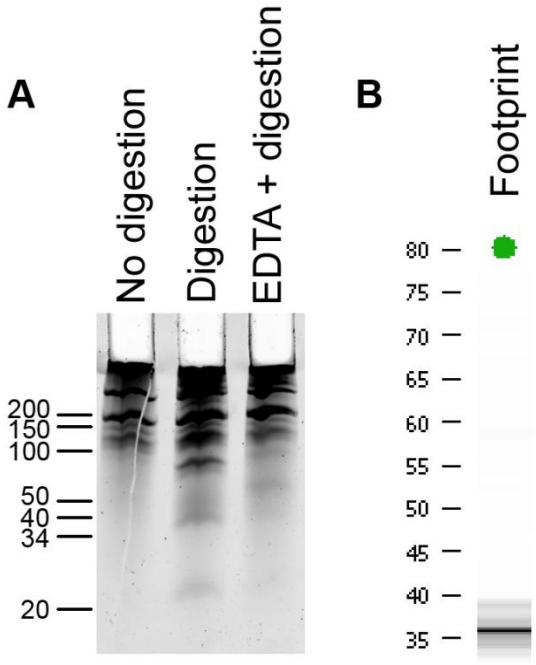


Figure 9: Isolation and characterization of ribosome footprints.

(A) Cells were treated with cycloheximide then lysed with 2% DDM. Indicated samples were treated with 10 mM EDTA for 10 minutes. Samples were digested with MNase and footprints isolated by acrylamide gel electrophoresis. (B) The size profile of an excised ribosome footprint as assessed by BioAnalyzer.

Deep sequencing reads representing ribosome footprints as well as total mRNA content in the cytosolic and ER compartments were mapped to RefSeq mRNAs (Pruitt et al., 2007). In Figure 10A, the distribution of mRNAs between the cytosol and ER compartments is plotted and identifies two distinct overlapping populations: one cytosol-enriched and one ER-enriched. The overall subcellular distributions are similar to those reported previously in cDNA microarray-based studies (Diehn et al., 2006; Mueckler and Pitot, 1981). Notably, the cytosol enriched mRNA population was

substantially represented on the ER, a finding that mirrors earlier observations on the relative population identities of the cytosolic and ER-associated mRNAs (Chen et al., 2011; Mueckler and Pitot, 1981). In the absence of information regarding the ribosome loading status of mRNAs in both compartments, the functional consequences of such mRNA distribution patterns are unknown. In the following, we examine the subcellular organization of mRNA translation through genome-scale analyses of ribosome loading in the cytosol and ER compartments.

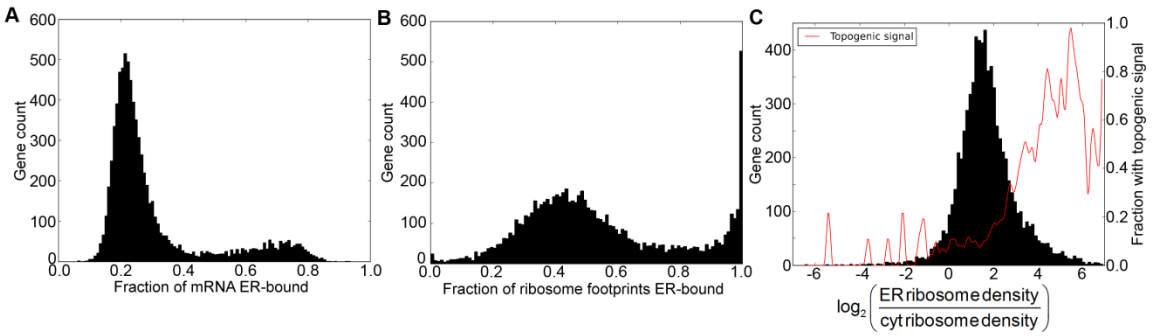


Figure 10: Subcellular distribution of mRNAs and translation.

A, histogram distribution of the relative enrichments of mRNAs on the ER. B, histogram distribution of the fraction of translation of each mRNA occurring on the ER. C, histogram distribution of the relative ribosome loading density for each mRNA in the cytosol (cyt) and ER compartments. Also plotted in C is a moving average of the fraction of transcripts that encode a transmembrane domain or signal sequence, as predicted by TMHMM or SignalP.

To survey the subcellular disposition of ribosome loading onto mRNAs, ribosomal footprint reads from the cytosol- and ER-derived sequencing libraries were counted to yield a measure of transcript-specific ribosome density in each compartment. As shown in Figure 10B, the subcellular distribution of ribosome density was markedly

biased to the ER compartment (Fig. 3A vs. 3B), with several mRNAs loaded with ribosomes almost exclusively on the ER. Ribosome loading density, defined as the ribosome footprints per mRNA transcript, translation was also found to be ER-biased (Figure 10C), indicating that most mRNAs are more densely loaded with ribosomes when associated with the ER. Of note, those mRNAs that are most efficiently loaded on the ER relative to cytosol are more likely to encode signal sequences and transmembrane domains, suggesting that the relative compartmental efficiency of translation varies for specific populations of mRNAs

To assess subcellular distributions of mRNAs and their compartment-specific translational status, cumulative density functions of mRNA abundance, number of ribosomes per mRNA, and ribosome loading density in the cytosol and ER compartments were determined. A broad distribution, ca. 4 orders of magnitude, was observed for each parameter. The cytosol and ER diverge in the relative abundance of each metric. In particular, as a population, mRNAs are more abundant in the cytosol, indicating that this compartment serves as the primary subcellular locale for the majority of transcripts (Figure 7D,Figure 10A,Figure 11A). The number of ribosomes bound to each species of mRNA is largely similar between the two compartments and the cytosol contains more mRNAs with relatively low quantities of associated ribosomes (Figure 11B). The ribosome loading per mRNA molecule in each compartment indicates, on average, substantially higher loading of ribosomes on ER-bound mRNAs, which in

agreement with the biochemical data presented in Figure 7, suggests that the ER is a preferred subcellular locale for ribosome loading (Figure 11C).

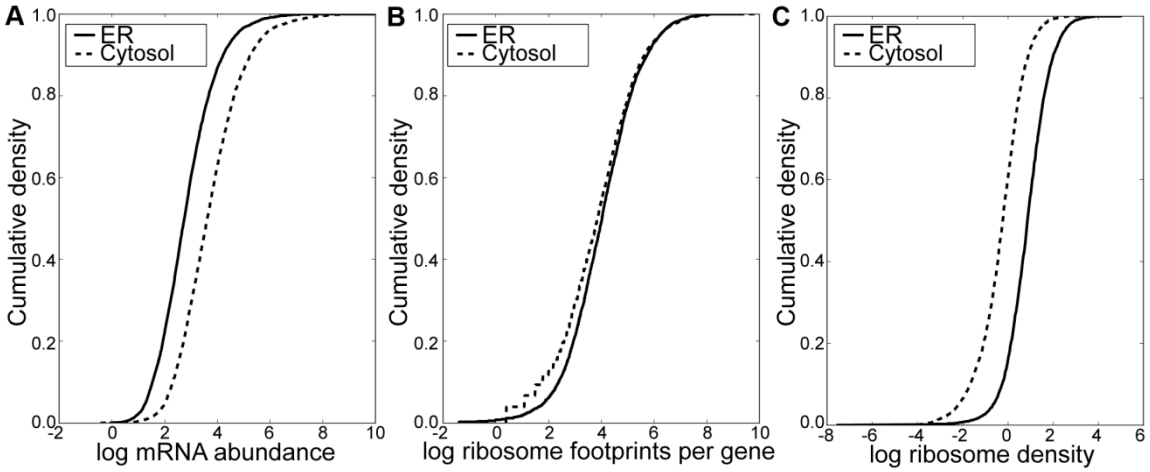


Figure 11: Cumulative density plot of subcellular mRNA abundance and translation.

A–C, cumulative density plots in the cytosol and ER for the abundance of each mRNA in the cytosol and ER (A), ribosomal footprints mapping to each transcript (B), and the density of ribosomes per mRNA for each gene (C).

3.3 Compartment-specific translational selection of mRNAs.

We investigated the nature of the subcellular ribosome loading bias through the lens of two primary cohorts of mRNAs: those encoding cytosolic proteins (i.e., lacking topogenic signals and functioning in the cytosol or nucleus) and those whose translation products are targeted to the ER (i.e., bearing topogenic signals and targeted to cellular membranes or for secretion). By this analysis, cytosolic-encoding mRNAs were largely localized to the cytosol, though as previously noted, this population was ca. ~20% represented on the ER (Figure 12A). Topogenic signal-encoding mRNAs were substantially ER-enriched, though with a small population displaying a cytosol

enrichment (Figure 12B). While the global subcellular mRNA distribution is largely consistent with previous reports (Chen et al., 2011; Diehn et al., 2006), the distribution of ribosome loading for both the cytosol and ER cohorts was substantially shifted to the ER compartment. The ribosome loading of cytosolic protein-encoding mRNAs displayed a broad distribution, with 20-80% occurring on the ER and the peak gene density occurring at ~45% translation on the ER (Figure 12C). With but few exceptions, mRNAs encoding ER-targeted proteins were loaded with ribosomes almost exclusively on the ER (Figure 12D).

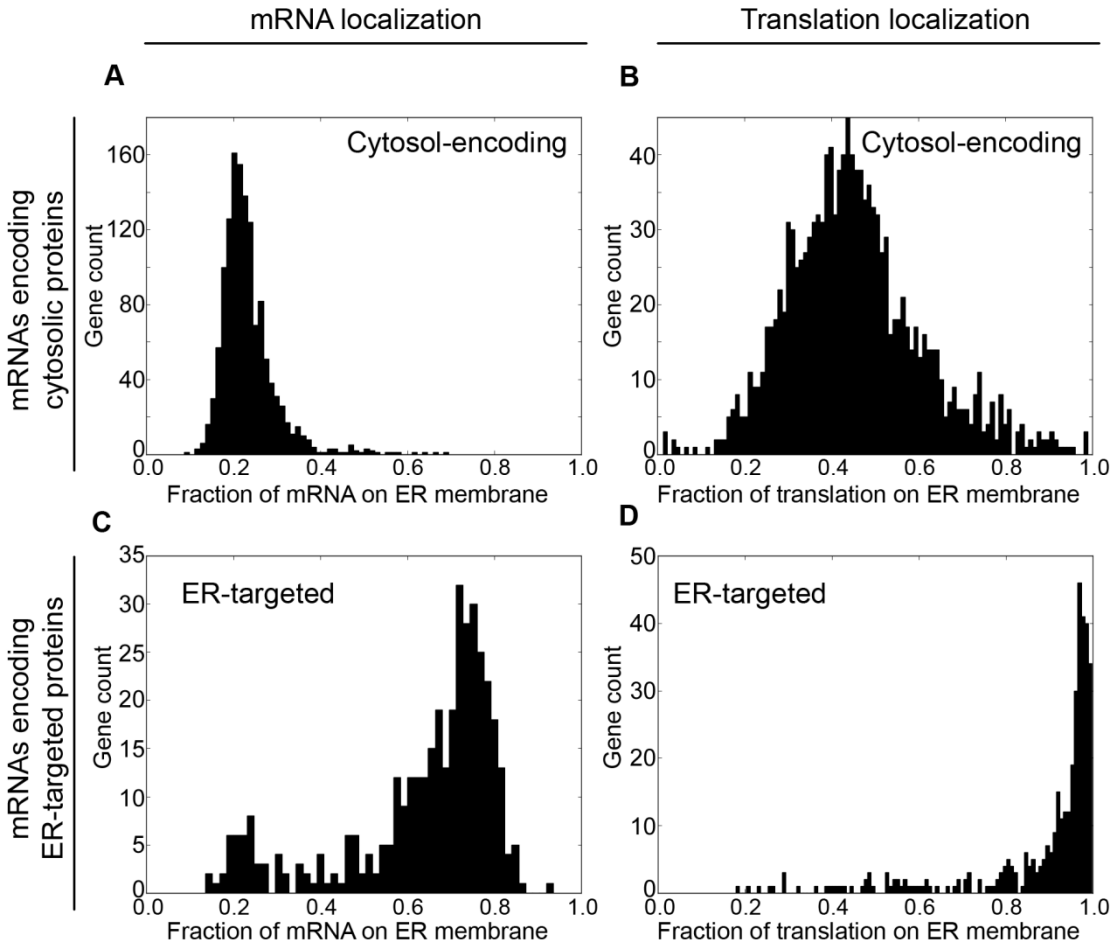


Figure 12: Subcellular mRNA partitioning and ribosome loading patterns of different mRNA cohorts.

A–D, histogram distributions of the fraction of ER-associated mRNAs for cytosolic protein-encoding (A) and topogenic signal-encoding mRNAs (B) and the fraction of ribosome loading on the ER for cytosolic protein-encoding (C) and topogenic signal-encoding (D) mRNAs.

To examine compartment-specific properties of translation, mRNA abundance and ribosome density for these two gene categories in the cytosol (Figure 13A) and ER (Figure 13B) were examined. In the cytosol, mRNAs encoding ER-targeted proteins are substantially less abundant and are sparsely loaded with ribosomes, indicating that

translation is significantly less robust for this cohort in the cytosol than for their counterparts encoding cytosol-targeted proteins. In contrast, mRNAs encoding both cytosol-targeted and ER-targeted proteins are similarly abundant and loaded with ribosomes when associated with the ER, suggesting that ribosomes in this compartment contribute to the synthesis of all cellular proteins. This pattern in reinforced in Figure 13C, where ribosome loading densities for these two cohorts are similar on the ER, but divergent in the cytosol. A strong positive correlation between ribosome loading in the two compartments for mRNAs encoding cytosolic proteins was observed, suggesting that the translational regulatory machinery may be shared. This correlation is substantially weaker for mRNAs encoding ER-targeted proteins. We also examined the relationship between mRNA localization and the localization of ribosome loading and observed that for mRNAs encoding cytosolic proteins, there is no substantial correlation between the two variables, suggesting that the localizations of mRNAs and their translation may be regulated separately (Figure 13D). In contrast, there is a significant positive correlation for mRNAs encoding ER-targeted proteins. Each of the divergences discussed here were statistically significant.

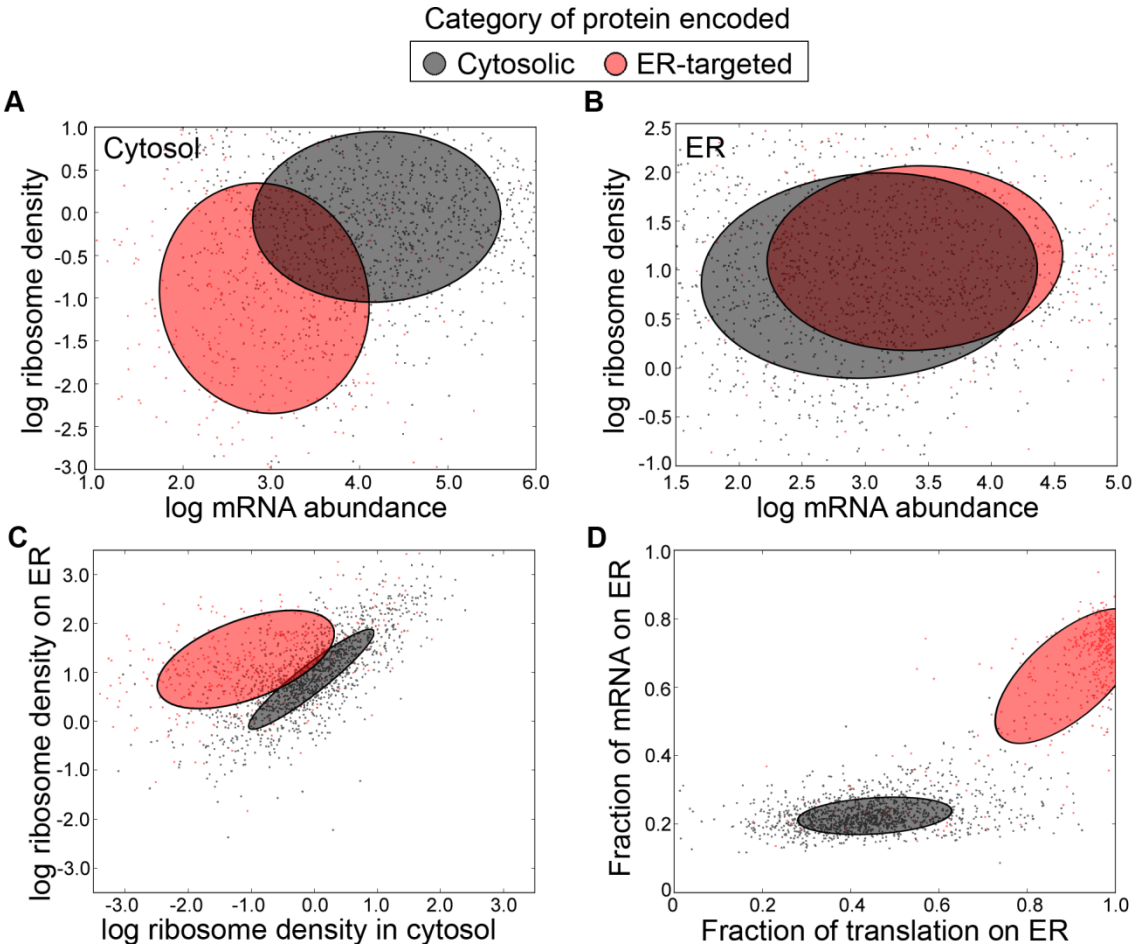


Figure 13: Divergent patterns of subcellular ribosome loading for different mRNA cohorts.

A and B, the abundance of each mRNA plotted against its loading with ribosomes in the cytosol (A) and ER (B). C, the density of ribosomal reads per mRNA in the cytosol plotted against that in the ER. D, the fraction of associated ribosomes for each mRNA on the ER plotted against the fraction of that mRNA present on the ER. In each plot, mRNAs encoding proteins targeted to the cytosol and ER are colored separately. Ellipses represent a 50th percentile density, and points represent individual mRNAs.

To characterize the nature of cytosolic-protein encoding mRNAs that are preferentially loaded with ribosomes in the cytosol or the ER, the enrichment of gene ontologies (GOs) was examined as a function of ribosome loading density in either

compartment. The analysis controls for the relative abundance of mRNAs in each compartment and so is only sensitive to differences in ribosome loading. The cohort of mRNAs encoding cytosolic products that were more heavily loaded with ribosomes on the ER was enriched for regulatory and dynamic cell functions, particularly the cell cycle. For example, the cell cycle regulators p53 (Matlashewski et al., 1984) and Myc (He et al., 1998) were both 4.5-fold more heavily loaded with ribosomes on the ER compared to the cytosol.

Several of the GO-defined cohorts that were efficiently translated in the cytosol were found to be associated with biochemical functions of relevance to plasma membrane function. For example, mRNAs encoding ICK, a protein kinase that localizes to basal membranes of epithelia (Togawa et al., 2000), are 4-fold more ribosome-loaded in the cytosol. These GOs may represent instances of mRNAs that are translationally activated in particular regions of the cell where their protein products are functional, in this case the areas proximal to the plasma membrane, which is consistent with current views on coupled mRNA localization and translational regulation (Martin and Ephrussi, 2009).

3.4 The ER and cytosol diverge in their spatial patterns of ribosome loading.

In light of the distinct patterns of compartment-enriched ribosome loading noted above and their potential repercussions regarding localized translation, we analyzed the

ribosome mapping dataset to derive insight into stage- (initiation, elongation, and termination) specific translational regulation and how this might diverge between the cytosol and ER compartments. This analysis assumes that read density is a representation of occupancy time and therefore the relative kinetics at each position. Total read density was plotted relative to the start and stop codon in the cytosolic (Figure 14A) and ER-associated mRNAs (Figure 14B) Both compartments display patterns in which ribosome density increases at the start codon and decreases at the stop codon, consistent with previous ribosome profiling studies (Guo et al., 2010; Ingolia et al., 2009). Also apparent is a three-base periodicity to the density, indicating that the single-codon procession of the ribosome was accurately captured. The cytosolic compartment displayed a clear density enrichment near the stop codon, suggesting that termination may be slower in the cytosol relative to the ER.

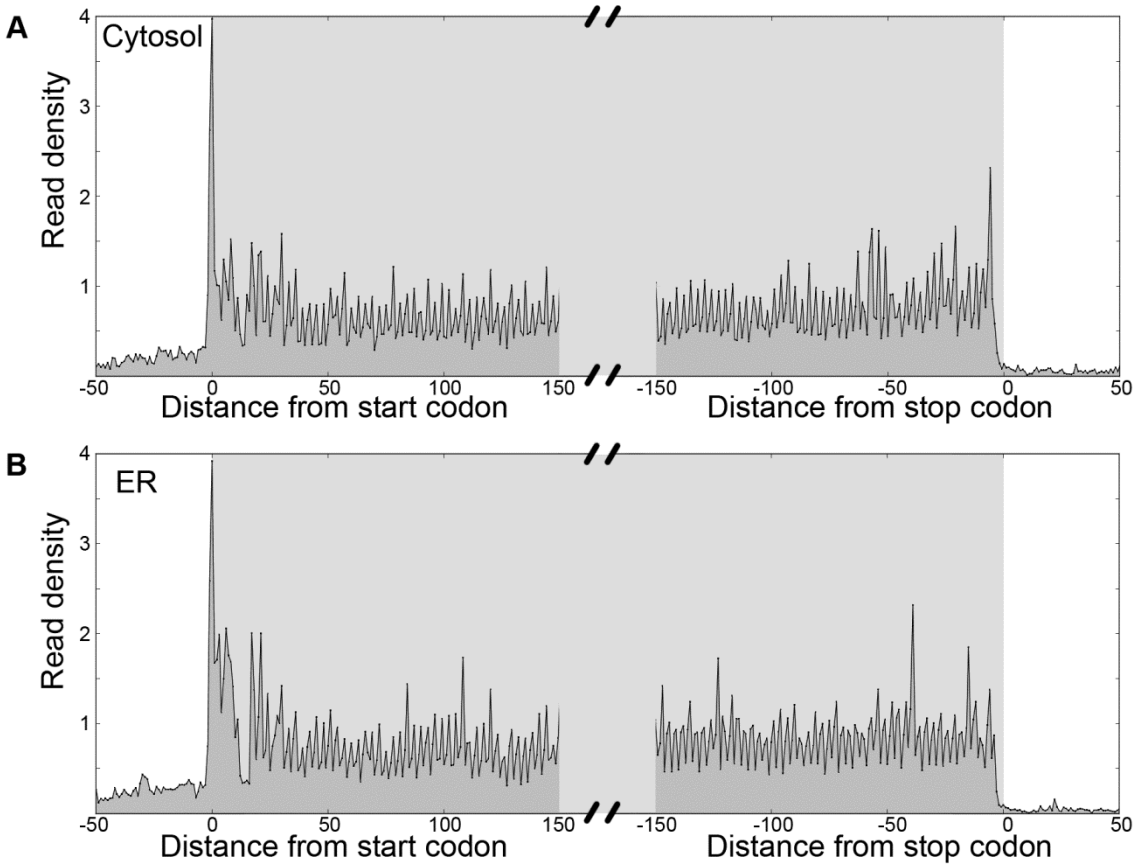


Figure 14: Spatial patterns of ribosome occupancy on mRNAs.

A and B, density of ribosomes near the start and stop codon for cytosolic (A) and ER (B) compartments.

The processivity of elongation was calculated by monitoring the density of ribosomes as a function of coding sequence position. This analysis suggested that translation in the cytosol was less processive than that in the ER (Figure 15), with decay constants of 0.00048 and 0.00019, respectively. These constants correspond to an average ribosome translational lifetime of 2083 amino acids in the cytosol and 5268 on the ER - much longer than average proteins. We hypothesized that this disparity could lead to

enrichment of translation of longer mRNAs on the ER, but no such trend was apparent. An alternative explanation is that ribosomes on the ER experience accelerated elongation rates over time relative to cytosolic ribosomes. Together, these analyses suggest that the composite biochemical reactions of translation in the cytosol and ER possess kinetic differences.

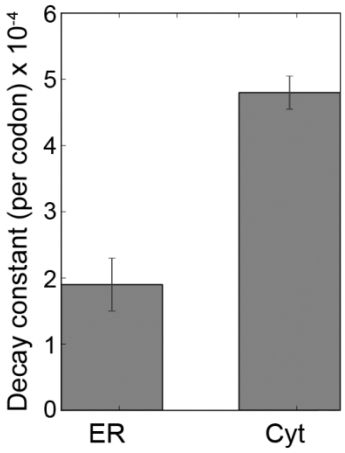


Figure 15: Ribosome processivity differs between cytosolic and ER-bound ribosomes. Ribosome processivity was calculated by fitting an exponential decay curve to the ribosome density in each compartment. Error bars represent at 95% confidence interval.

It is also notable that the cytosol and ER both have similarly large peaks of ribosome density at the start codon. The high density in both compartments indicates that translational initiation occurs to a similar degree in both cellular compartment – a finding that stands in contrast to the basic SRP model, where all initiation occurs on cytosolic ribosomes. The localization of initiation to the ER indicates that both ribosomes and mRNAs may be stably localized to the ER, completing an entire cycle of translation

without at any point being released to the cytosol. Stable binding of mRNAs and ribosomes to the ER would allow for long-term divergent regulation of cytosolic and ER-bound mRNAs and ribosomes.

3.5 The ER as a general site for translation

In the current study, we have used ribosome profiling to investigate the *in vivo* role of the ER in mRNA translation and report two primary observations: 1) mRNAs encoding cytosolic proteins were broadly loaded with ribosomes on the ER and 2) steady-state ribosome loading on ER-bound mRNAs was substantially higher than in the cytosol. In demonstrating that ER-localized mRNAs display higher ribosome loading than their cytosolic counterparts, these findings expand the landscape of post-transcriptional regulation of gene expression to include the partitioning of mRNAs between the cytosol and ER compartments. Curiously, subcellular translational status was independent of the localization of the mRNA itself, indicating that mRNA localization and translational activity comprise two separately-regulated processes. The importance of these variables is underscored by the enhanced ribosome loading on the endoplasmic reticulum for mRNAs involved is a wide range of regulatory and dynamic cellular functions. These observations suggest that the ER may serve as a preferred locale for the synthesis of proteins involved in, for example, the cell cycle. Combined with the previously reported enhanced half-life of ER-associated mRNAs (Hyde et al., 2002) and the relative absence of ER-bound mRNA trafficking into stress granules (Unsworth et al., 2010), the sum of

findings to date point towards a global role for the ER in the expression of the transcriptome.

At present, the molecular basis for the regulation of ER-compartmentalized translation is unknown. One hypothesis for the divergent translational efficiencies of the two compartments is that RNA binding proteins, many of which modulate a wide range of post-transcriptional processes (Mansfield and Keene, 2009) and confer compartment-specific translational efficiency (Besse and Ephrussi, 2008) are compartmentally segregated between cytosolic and ER-bound polysomes. Indeed, it is plausible that one or more of the many RNA binding proteins shown to change translational status of mRNAs operate by modulating translation in a compartment-specific manner. A related mechanism was recently described in the case of RPL38, a ribosomal protein that promotes translation of a specific subset of Hox genes (Kondrashov et al., 2011).

Interestingly, despite ribosome footprinting indicating a far greater average ribosome density in the ER, the cytosol and ER polyribosome profiles, as assessed by sucrose gradient fractionation, are similar. This divergence indicates that there may exist a substantial pool of mRNAs that are not ribosome associated, and that this pool is largely segregated to the cytosol. Indeed, it has been demonstrated that ca. 25% of all yeast mRNAs are not ribosome-associated (Arava et al., 2003). Should these mRNAs be predominantly localized to the cytosol, perhaps associated with processing bodies or stress granules, this would indicate a similar ribosome loading density in each

compartment for those mRNAs that are available for translation. Given that ER-bound mRNAs are resistant to recruitment into stress granules (Unsworth et al., 2010), it is plausible that compartmental differences in stress granule and processing body association lead to a large population of cytosol-localized mRNAs that are not associated with polyribosomes. This model is consistent with Figure 8A, where mRNAs in the cytosol are more frequently not associated with polysomes than those on the ER. The ER may therefore represent a cellular compartment for efficient protein synthesis, with the cytosol performing the ancillary functions of mRNA storage, degradation, and related processing.

This chapter describes the existence of two functionally distinct compartments for protein synthesis. While the functional ramifications and mechanistic details remain to be developed in following chapters and elsewhere, we suggest that the choice between translation in the cytosol or on the ER represents a fundamental division in post-transcriptional regulation with broad consequences for the resulting proteome.

Methods

Cell Culture and Fractionation

HEK293 cells were grown in DMEM supplemented with 10% FBS. To purify cytosolic and ER-bound ribosomes, cells were grown to ~70% confluence and harvested in PBS. Cells were collected by centrifugation and resuspended in an isotonic cytosol buffer solution containing 0.03% digitonin to release the cytosol contents. Digitonin-

permeabilized cells were subsequently washed with cytosol buffer containing 0.015% digitonin, and the ER-associated polyribosomes were released by the addition of an ER lysis buffer containing 2% n-dodecyl-ß-d-maltoside. All solutions contained 180 μm cycloheximide, 1 mm dithiothreitol, and 40 units/ml RNaseOUT (Invitrogen).

Polysome Gradient Fractionation

Cytosol and ER subcellular fractions were overlaid onto 10-ml 15–50% linear sucrose gradients containing 400 mm KOAc, 25 mm K-HEPES, pH 7.2, 15 mm Mg(OAc)2, 180 μm cycloheximide and centrifuged for 3 h at 151,000 × g in a Beckman SW-41 Ti rotor. Gradients were harvested with a Teledyne Isco gradient fractionator with continuous A260 monitoring. For each experiment, a paired blank gradient containing only lysis buffer was run in parallel, and the A260 profile was used for background subtraction. For radiolabeling experiments, cell cultures were supplemented with 200 μCi/ml [35S]Met/Cys (MP Biomedical) for 2 min, treated with either cycloheximide (180 μm final concentration) or puromycin (250 μm final concentration), and fractionated as described above, and ribosomes were recovered by centrifugation (15 min, 85,000 rpm, TLA100.3 rotor) over a 35% sucrose cushion.

Semiquantitative PCR

RNA from gradient fractions was reverse transcribed using M-MLV reverse transcriptase and then amplified using the following primers: GAPDH, 5'-AGCCACATCGCTCAGACAC-3', 5'-GCCCAATACGACCAAATCC-3'; ACTB, 5'-

AGAGCTACGAGCTGCCTGAC-3', 5'-CGTGGATGCCACAGGACT-3'; GRP94, 5'-CTGGAAATGAGGAACTAACAGT-3', 5'-TCTTCTCTGGTCATTCCTACACC-3'. PCR amplicons were analyzed by agarose gel electrophoresis and visualized using SYBR Green (Invitrogen).

Analysis of Compartmental Translational Profiles

To determine the subcellular distribution of mRNA-associated ribosomes, cells were biosynthetically labeled for 48 h with 6 μCi/ml 5,6 [3H]uridine (MP Biomedicals) and fractionated as above. The buffer composition of each fraction was adjusted to 500 mm KOAc, 25 mm K-HEPES, pH 7.2, 15 mm Mg(OAc)2, 180 μm cycloheximide. Extracts were incubated with oligo(dT)7 resin (Amersham Biosciences) at 40 mg/ml for 1 h at room temperature and centrifuged, and the resin washed extensively in loading buffer and subsequently isolated on Micro Bio-Spin chromatography columns (Bio-Rad). Oligo(dT)7 resin was then eluted with 1 ml of 500 mm KOAc, 15 mm EDTA, 25 mm K-HEPES, pH 7.2. Relative ribosome levels were determined by liquid scintillation spectrometry of the EDTA eluates. To obtain mRNA levels, purified RNA pools were treated with RiboMinus (Invitrogen) according to the manufacturer's protocol, and poly(A) mRNA abundance was assessed by Bioanalyzer (Agilent).

Ribosome Footprint Preparation, Sequencing, and Mapping

Cell fractions were adjusted to 100 mm KOAc, 25 mm K-HEPES, pH 7.2, 15 mm Mg(OAc)2, 3 mm CaCl2, 180 μm cycloheximide and treated with 20 μg/ml micrococcal

nuclease (Sigma-Aldrich) for 1 h at 37 °C. Nuclease activity was inactivated by the addition of 6 mm EGTA, and ribosomes were recovered by centrifugation (60 min, 90,000 RPM in the Beckman TLA 100.3 rotor) over a 500 mm sucrose cushion. The purified ribosome pellet was resuspended in 50 mm sodium chloride, 50 mm Tris, pH 7.5, 5 mm EDTA, 0.5% SDS, 200 μg/ml proteinase K and incubated for 30 min at 37 °C (26). RNA was purified by phenol/chloroform extraction and separated on a 15% acrylamide gel containing 1× Tris-borate-EDTA and 7 m urea. Gels were stained with SYBR Gold (Invitrogen), revealing an ~35-nt band that was absent when ribosomes were dissociated with EDTA prior to nuclease digestion (supplemental Fig. S1). This region of the gel was excised, and RNA was extracted by homogenizing the gel in 100 mm NH4HCO3, freezing to -80 °C, rapidly reheating to 95 °C, and incubating the gel homogenate, with mixing, for 3 h at room temperature. Acrylamide fragments were removed by centrifugation, and the remaining supernatant fraction was filtered through a SpinX filter (Costar) by centrifuging for 10 min at 12,000 × g. RNA was recovered by EtOH/NaOAc precipitation.

cDNA libraries were prepared for SOLiD sequencing with the Applied Biosystems Small RNA protocol by the Duke University Genome Sequencing & Analysis Core Resource facility. Briefly, the purified 35-nt RNA pool was ligated to oligonucleotide adapters and reverse transcribed. Fragments were then amplified by PCR, size-selected, and applied to beads, which were deposed onto a chip surface for

sequencing. Each bar-coded sample was sequenced using the SOLiD 4 system, obtaining ~115 million 35-base reads between the two samples.

Sequencing of Total mRNA from Cellular Compartments

From the same cellular fractions as were used for ribosome footprinting, a sample of total RNA was purified by TRIzol extraction. Total RNA samples were treated with RiboMinus (Invitrogen) and prepared for bar-coded SOLiD sequencing according to the manufacturer's instructions, where total mRNA was treated with RNase III and prepared for sequencing as described above. In this run, 35 million 50-base reads were obtained between the two samples.

Read Mapping to mRNAs

Total mRNA reads were initially mapped to all RefSeq RNAs (hg19, GRCh37), allowing for 10 maps per read to encompass the multiple annotated isoforms that exist for several genes (27). To select a single mRNA to represent each gene, the longest annotated mRNA with an mRNA read density of at least 75% of the maximum read density was chosen. Reads from mRNA and ribosome footprinting libraries were then remapped to this subset of mRNAs, allowing each read to map to two locations, allowing two mismatches in a 25-nt seed region, and enabling the best and strata options. To ensure that the RefSeq database provides a thorough representation of the HEK293 transcriptome, the density of reads that span exon-exon junctions was compared with those that do not; no appreciable differences were observed.

Total mRNA libraries were normalized by the compartmental abundance of mRNA (see Fig. 1C), and ribosome footprinting libraries were normalized by the abundance of poly(A)-associated ribosomes (see Fig. 1D). All transcripts with at least 10 total mRNA reads per kilobase per million reads were considered. The abundance of each species of mRNA or its translation was estimated by the number of reads mapping to that mRNA per million mapped reads normalized by the length of that mRNA (for total mRNA) or coding sequence length (for ribosome footprints) in kilobases (21). Finally, to assess ribosome loading per mRNA, or ribosome density, ribosome loading was divided by the abundance of that mRNA. The cytosol and ER-targeted gene categories were described previously (9).

Analysis of Ribosome Density Distribution

To evaluate the density of ribosome footprints, the number of reads that span each position relative to the start and stop codon was counted and normalized by total read number. For positional analyses, the ribosome position was taken to be the start of the read +15 nt, which maps to the A site of the ribosome (22). To calculate the processivity of ribosomes in each compartment, the density of ribosomes from 200 to 800 nt to the 3' of the start codon was counted for all mRNAs with a coding sequence of >1100 nt. A best-fit line was then fitted to the natural log of the smoothed density.

Calculation of Gene Ontology Enrichment

Gene ontology (GO) enrichments were calculated by permutation testing. The mean relative translational efficiency, log2(ER ribosome loading/cytosol ribosome loading), for each gene ontology category was calculated and compared with 10,000 nulls in which gene values were randomly permuted. Significance was determined using Benjamini-Hochberg multiple hypothesis correction from a p value cutoff of 0.05.

4. Reprogramming and recompartmentalization of translation in the Unfolded Protein Response

Having established the ER plays a major role in the synthesis of proteins throughout the cell, we sought to characterize how this compartmentalization may be modified by the cell in the context of stress responses. We identified a dramatic recompartmentalization of translation upon induction of ER stress, demonstrating that mRNA and translation localization can be an important component of cellular signaling programs. We further begin to characterize the various components that can regulate mRNA and translation localization.

Parts of this chapter are currently in review at Cell. Qiang Chen performed isotopic labeling experiments and Angeline Su Ling performed Western blots.

4.1 The architecture of translation in cellular stress response

Protein synthesis is tightly coupled to protein homeostasis so that proteome function can be maintained during environmental and physiological stress. Among the most rapid

and specific means to this end is the reprogramming of translational activity (Mata et al., 2005). One such program, the Unfolded Protein Response (UPR), is initiated by the accumulation of unfolded proteins in the endoplasmic reticulum (ER) (Walter and Ron, 2011). By acting to both reduce the protein folding load of the ER and increase the organelle's protein folding capacity, the UPR provides a conserved mechanism for responding and adapting to proteostatic stress.

The UPR stress response program operates within the context of a translational machinery (ribosomes, mRNAs, etc) that is compartmentalized between the cytoplasm and endoplasmic reticulum (ER) (Chen et al., 2011; Palade, 1956; Voeltz et al., 2002). In a typical cell, half of all ribosomes and a third of all mRNAs are stably associated with the ER membrane (Reid and Nicchitta, 2012). Both mRNAs and ribosomes can be tethered to the ER independently of one another (Chen et al., 2011; Seiser and Nicchitta, 2000). One function of this compartmentalization is to allow the co-translational insertion of membrane and secretory proteins into the ER lumen, thus initiating the secretory pathway (Palade, 1975). In addition, a substantial fraction of all mRNAs encoding cytoplasmic proteins is localized to and translated on the ER, making the ER a primary site for the synthesis of the proteome generally (Diehn et al., 2000; Reid and Nicchitta, 2012). This large-scale compartmentalization within the cell opens the possibility that localization may be an important control point in translational regulation, particularly with respect to the findings that the activity of cytoplasmic and ER-associated ribosomes

can be regulated independently (Stephens and Nicchitta, 2008).

The UPR, a stress response program that is also a process that is also highly compartmentalized, couples translation to the protein folding status of the endoplasmic reticulum (ER). The primary mechanism by which the UPR modifies translation is through the phosphorylation of eIF2α at Ser51 by PERK, an ER transmembrane eIF2α kinase that senses the folding state of proteins in the ER and transmits that signal to the cytosol (Harding et al., 1999; Prostko et al., 1993). Phosphorylation of eIF2α renders eIF2 inactive, resulting in a general suppression of translational activity. PERK activity is opposed by two phosphatase-activating proteins that have distinct subcellular localizations: CReP (*Ppp1r15b*), an ER-associated protein, and a cytosolic protein GADD34 (*Ppp1r15a*) (Connor et al., 2001; Kloft et al., 2012; Zhou et al., 2011). Because the UPR is activated in response to a highly compartmentalized stress (only ER protein folding is perturbed), we reasoned that the UPR may remodel the activity of cytosolic and ER-associated polyribosomes, and of translation of ER-targeted and cytosolic proteins, in distinct ways.

Here, we find that the UPR drives a large-scale recompartmentalization of translation between the cytosol and ER, where mRNAs that encode membrane and secreted proteins are rapidly released from the ER upon induction of stress. Further, we find that this translational response is modulated in compartmentally specific manners by GADD34 and CReP. These findings demonstrate that the dynamic relocalization of

mRNAs and translation between the cytosol and ER can serve as a rapid, selective means of translational regulation during cell stress and recovery.

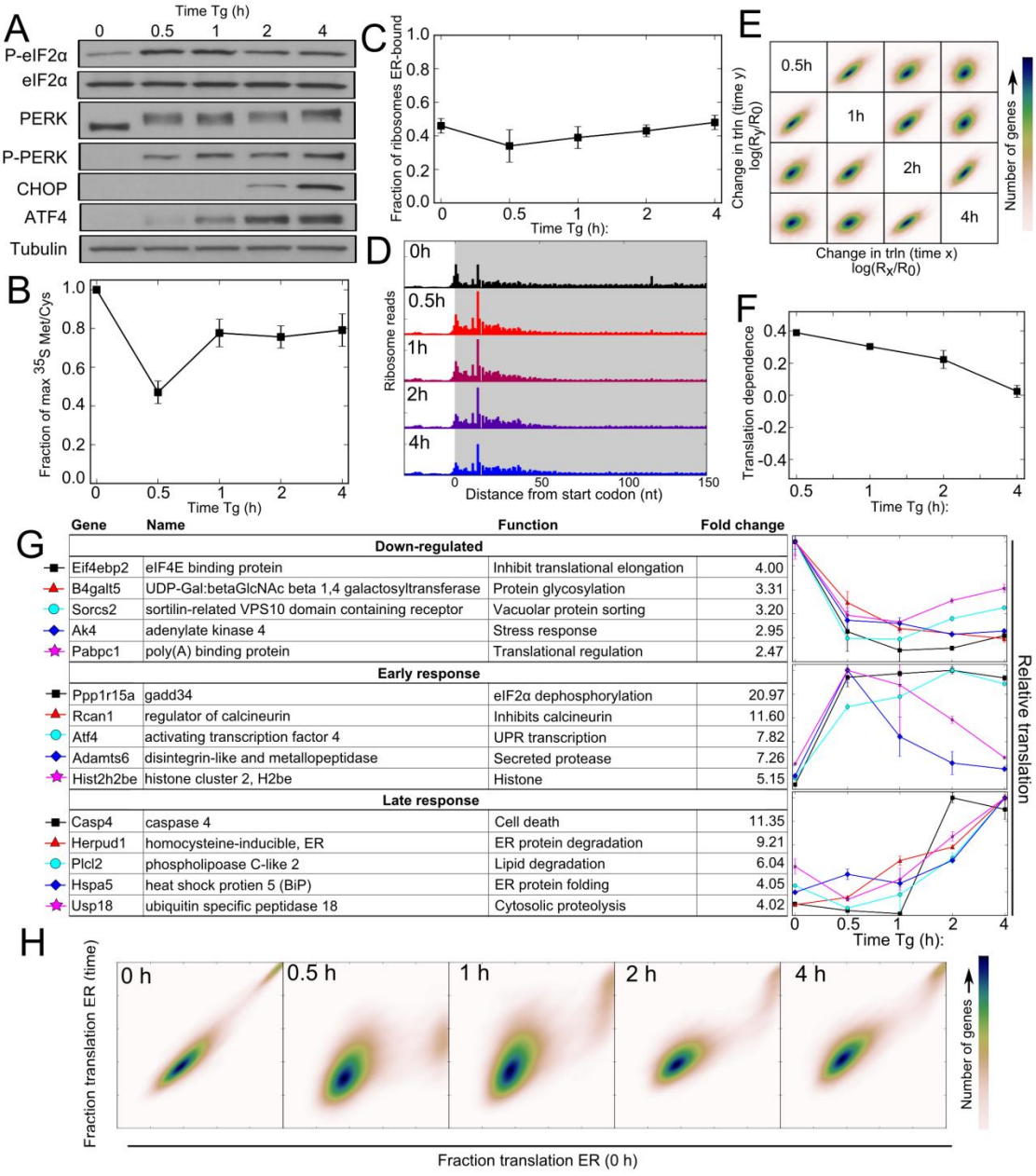
4.2 Translational profiling in the UPR

To analyze the how translation is remodeled in the UPR, we treated mouse embryonic fibroblasts (MEFs) with 1 μM thapsigargin (Tg), which elicits ER protein folding stress by inhibition of SERCA (Wuytack et al., 2002), over a timecourse from 30 min to 4 h. Over this period, eIF2α was initially heavily phosphorylated, then gradually recovers to near its initial low phosphorylation state over time (Figure 16A). The eIF2α kinase PERK was increasingly activated by phosphorylation, while CHOP and ATF4, two UPR-related transcription factors, were induced by late time points. Total translational activity as measured by [^{35}S]Met/Cys incorporation was reduced by ~50% at 0.5 h, then mostly recovered, although on a timecourse distinct from eIF2α phosphorylation. Instead of a gradual recovery, total translation rapidly returned to 80% of untreated cells, then plateaued. This recovery occured despite the ongoing presence of Tg and continued PERK activation (Figure 16A), and by 4 h, the cells had adapted a new, Tg-induced translational steady state. Having determined that this treatment period encompasses the detection, response, and adaptation to unfolded protein accumulation, we treated MEFs with Tg, separated cytosolic and ER-associated polyribosomes and analyzed them by ribosome profiling. These data provide a transcriptome-wide quantitative, nucleotide-resolution snapshot of ribosome position and density on

mRNAs in each cellular compartment (Ingolia et al., 2009). In parallel, we quantified ribosome compartmentalization by purifying ribosomes from each cellular compartment and quantifying by A_{260}, finding that some ribosomes are released from the ER in the UPR (Figure 16C). To define the localization of mRNAs, we purified poly-A mRNA from each compartment for analysis by RNA-seq. Together, these data define the localization of each mRNA, of its translation, and its density of ribosome loading over time.

In untreated cells (t=0 h), ribosomes were distributed in the coding sequence with a standard three-nucleotide periodicity with two exceptions: a large peak at the start codon and a large peak at 13 nt, each of which has been observed previously to varying degrees (Liu et al., 2013). Upon induction of the UPR, the pattern of ribosome positions on mRNAs changed such that ribosomes were clustered heavily in the first 50 nt of the coding sequence. The peak at 13 nt also increased as the peak at 0 nt decreased (Figure 16D). A similar profile was observed in treatments with non-natural amino acids, indicating that this may be a general feature of proteotoxic stresses, likely a defect in

translational elongation (Liu et al., 2013).



Gene	Name	Function	Fold change
Down-regulated			
Eif4ebp2	eIF4E binding protein	Inhibit translational elongation	4.00
B4galt5	UDP-Gal:betaGlcNAc beta 1,4 galactosyltransferase	Protein glycosylation	3.31
Sorcs2	sortilin-related VPS10 domain containing receptor	Vacuolar protein sorting	3.20
Ak4	adenylate kinase 4	Stress response	2.95
Pabpc1	poly(A) binding protein	Translational regulation	2.47
Early response			
Ppp1r15a	gadd34	eIF2α dephosphorylation	20.97
Rcan1	regulator of calcineurin	Inhibits calcineurin	11.60
Atf4	activating transcription factor 4	UPR transcription	7.82
Adamts6	disintegrin-like and metallopeptidase	Secreted protease	7.26
Hist2h2be	histone cluster 2, H2be	Histone	5.15
Late response			
Casp4	caspase 4	Cell death	11.35
Herpud1	homocysteine-inducible, ER	ER protein degradation	9.21
Plcl2	phospholipoase C-like 2	Lipid degradation	6.04
Hspa5	heat shock protien 5 (BiP)	ER protein folding	4.05
Usp18	ubiquitin specific peptidase 18	Cytosolic proteolysis	4.02

Figure 16: Disruption and recovery of translation in the UPR.

(A) Western blots against UPR-associated factors (B) Timecourse of translational activity during Tg treatment as measured by [^{35}S]Met/Cys incorporation. (C) Distribution of ribosomes between the cytosol and ER (D) Distribution of ribosome positions relative to

the start codon at each experimental time point. (E) Heatmap comparing the changes in translation at each timepoint for each mRNA. Correlation coefficients are indicated in the lower right of each panel. (F) Median dependence of all mRNAs on changes in translational efficiency relative to mRNA concentration to give rise to a change in total amount of translation relative to Control. Here, a score of 0 indicates equal reliance on translation and mRNA concentration, higher values indicate translation dependence, and lower values indicate mRNA dependence. (G) Selected genes from each stage of the UPR, defined as: down=R_0/avg($R_{0.5}$…R_4); early=$R_{0.5}$/R_0; late=R_4/avg(R_0,$R_{0.5}$). The trajectories of each gene's translation is indicated to the right of each table. (G) Comparison of translational localization for each mRNA at each time point relative to Control. In the 0 h panel, biological replicates are compared to assess experimental error.

We quantified the transcript-level changes in translation that occur during the UPR by counting the length-normalized number of ribosome footprinting reads in each transcript's coding sequence, which we define as R. Each time point of Tg treatment induced a translational response that diverges widely from experimental error. The translational response to Tg changed significantly over time; following a large change in translation after 0.5 h treatment, the response continued to evolve such that it has almost no resemblance to that at 4 h (Figure 16E). Each time point closely resembled those close to it, while diverging almost entirely from more distant time points indicating that the UPR comprises a multiphasic program with distinct stages. These changes in total translation can be explained by two variables: the concentration of a particular mRNA and the efficiency of translation (ribosome loading) of that mRNA. To determine which of these variables is the primary driver of UPR-induced translational remodeling, we developed a $\log_2$-based metric, which we term translational dependence, to express the relative weights of translational efficiency and mRNA concentration in giving rise to the

observed amount of translation. At early time points, changes in protein expression were determined mostly by changes in translational efficiency, while mRNA levels were relatively constant (Figure 16F). At later time points, however, the UPR had shifted significantly in character, becoming equally reliant on transcriptional and translational changes. We therefore divided the UPR into three categories: down-regulated genes (reduced translation throughout Tg treatment), primarily translation-dependent early response (enhanced translation at the 0.5 h time point), and relatively mRNA-dependent late response (translation induced at 4h relative to control and 0.5 h).

In each of these three categories, a number of genes were highly responsive to UPR activation and provide clues to how the UPR modifies cellular activity to adapt to unfolded proteins (Figure 16G). Many mRNAs that were down-regulated encode proteins involved in diverse aspects of protein synthesis and maturation: translation, protein glycosylation, and secretion were all significantly affected. This likely contributes to decreasing the protein folding load in the ER. The translation of these mRNAs, however, remained low even as overall translation recovered substantially. This stable down-regulation may represent a component of a new steady state is established in response to the ongoing presence of Tg. Many mRNAs had significantly enhanced translation following the induction of the UPR. Of these transcripts, many encode previously identified UPR proteins such as Gadd34, Atf4, and Rcan1. Several such mRNAs encode proteins that point towards novel means the cell may use to adapt

to unfolded proteins. Adamts6 encodes a heavily-induced secreted protease. Also among the most strongly induced proteins were those that comprise the nucleosome; on average, their translation increases by 294%. Strikingly, the increase in translation of histones was accompanied by a major recompartmentalization of their translation to the ER. The role of this up-regulation in the UPR is unclear, although it may be relevant to the transcriptional component of the response. Even among those whose induction had previously been identified, the translational profiles of these proteins varied dramatically over time. Gadd34 and Atf4 were induced and remained high, while Rcan1 and Hist2h2be were induced and then dropped precipitously by 4 h. By the 4 h time point, a number of new proteins had also been induced. Several were associated with one well-characterized outcome of chronic UPR activation: apoptosis. Simultaneously, other induced proteins continued to be associated with increasing protein folding capacity while decreasing protein folding load, demonstrating two divergent strategies in late stress periods: recovery of ER homeostasis by means not exploited in early stress and preparation for cell death should that recovery fail. Together, these changes in translation demonstrate the diverse strategies to restore protein folding homeostasis in the face of continued protein folding stress.

4.3 Release of mRNAs encoding ER-targeted protein from the ER

Over the course of induction and response to unfolded protein stress, we observed another major reprogramming of translation: the compartmentalization of many

transcripts' translation was significantly and rapidly modified (Figure 16H). In untreated cells, most mRNAs were ~30-50% translated on the ER, with a second population – mostly mRNAs encoding membrane, organelle, and secreted (collectively, ER-targeted) proteins – translated almost exclusively on the ER. Upon induction of stress, there was a dramatic reorganization of this framework, with a large fraction of mRNAs that were ER-enriched moving to the cytosol and the distribution of the more cytosolic mRNAs tending more towards the ER. The disruption of localization recovered gradually over the timecourse, and by 4 h, the translation of most mRNAs had returned to the localization they possessed in resting cells. This large-scale reprogramming of the spatial distribution of translation could be an important aspect of the translational response to unfolded proteins, which was investigated further.

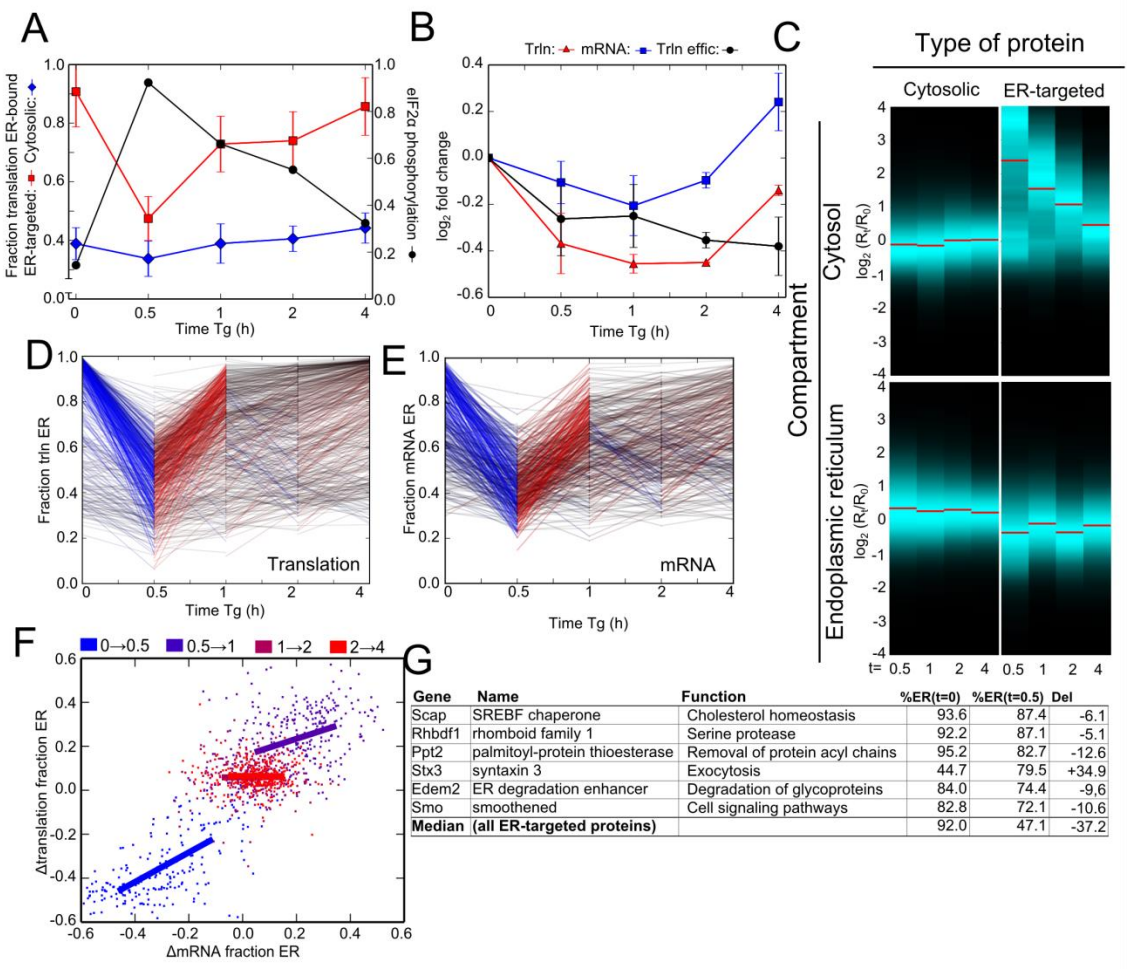
We examined mRNAs encoding ER-targeted proteins and mRNAs encoding cytosolic proteins separately, plotting the median localization of their translation over the treatment timecourse (Figure 17A). For mRNAs encoding ER-targeted proteins, the localization of their translation - over 90% ER-associated in resting cells - was significantly disrupted after 30 min Tg treatment, dropping by nearly a third before gradually regaining their ER localization. In contrast, the translation of mRNAs encoding cytosolic proteins was mostly unchanged. Curiously, this effect is well correlated with the phosphorylation status of eIF2α. The relocalization of ER-targeted protein translation was initially accompanied by a large decrease in their translation, but

no significant decrease in mRNA abundance (Figure 17B). This resulted in a regulatory landscape where translational efficiency (ribosomes per mRNA) was dominant over early time points, until the 4h time point, at which point the mRNAs encoding many ER-targeted proteins began to increase (Fig. 2B). Curiously, this decrease in translational efficiency did not recover as the translation of ER-targeted mRNAs returns to the ER at later time points. These data suggest that the release of translation of ER-targeted mRNAs is a short-term strategy the cell adopts while the translational regulatory apparatus prepares for a long-term down-regulation of ER-targeted proteins in order to decrease ER protein folding load.

We next asked whether the decrease in ER enrichment is driven by a decrease in translation on the ER, an increase in translation in the cytosol, or both. On average, the number of cytosolic ribosomes bound to mRNAs encoding ER-targeted proteins increased by over 400% at the peak of the translational response before returning to near steady state levels at 4h (Figure 17C). There was a corresponding decrease in translation of ER-targeted proteins on the ER at 0.5 h. This decrease represented, on average, a 1:1 exchange where loss of footprints on the ER gives rise to a corresponding increase in the cytosol (Figure 18A). In contrast, the translation of mRNAs encoding cytosolic proteins was mostly unchanged. This divergence further illustrates the large-scale recompartmentalization of translation from the ER to the cytosol that is highly selective for ER-targeted proteins.

When analyzing the localization trajectories of individual transcripts over time, we observed that, for ribosome bound to mRNAs encoding ER-targeted proteins, a large population of these transcripts is re-compartmentalized to the cytosol at 0.5 h, then returns to the ER by 1h as the translational phase of the UPR transitioned into the transcription-driven phase (Figure 17D). In general, the magnitude of release to the cytosol was mirrored by a recovery in ER localization of similar magnitude at 1 h. A similar shift was apparent was apparent for the mRNAs themselves, indicating that mRNAs recompartmentalize along with their translation (Fig. 2E). The shift of mRNAs and of their translation was well correlated during the initial recompartmentalization (Figure 17F). Because both the mRNA distribution and ribosome footprint reads transitioned in tandem, it appears that UPR activation elicits the release of mRNAs from the ER largely as intact polyribosomes. The slope of the best fit line, however, was less than 1 (0.67), indicating that on average, when a mRNA encoding an ER-targeted protein is released from the ER, 33% of its ribosomes retained ER association. This correlation broke down as the UPR shifted to its transcriptional phase. One surprising aspect of this shift is that there were any ribosomes bound to mRNAs encoding ER-targeted proteins in the cytosol whatsoever, as completed proteins would lack their normal ER folding environment and would likely aggregate. Curiously, mRNAs encoding ER-targeted proteins had indistinguishable ribosome positioning and density in the cytosol and ER, indicating either that these ribosomes were either stalled once

they are released from the ER or that protein synthesis continues despite the lack of proper localization (Figure 18B). In the latter situation, these proteins are likely to be degraded. Regardless, these results demonstrate that large, specific populations of mRNAs can be relocalized within the cell in response to specific stimuli. In this context, the removal of mRNAs encoding ER-targeted proteins from the ER likely aids in decreasing the compartment's protein folding load.



Gene	Name	Function	%ER(t=0)	%ER(t=0.5)	Del
Scap	SREBF chaperone	Cholesterol homeostasis	93.6	87.4	-6.1
Rhbdf1	rhomboid family 1	Serine protease	92.2	87.1	-5.1
Ppt2	palmitoyl-protein thioesterase	Removal of protein acyl chains	95.2	82.7	-12.6
Stx3	syntaxin 3	Exocytosis	44.7	79.5	+34.9
Edem2	ER degradation enhancer	Degradation of glycoproteins	84.0	74.4	-9.6
Smo	smoothened	Cell signaling pathways	82.8	72.1	-10.6
Median	**(all ER-targeted proteins)**		92.0	47.1	-37.2

Figure 17: mRNAs encoding ER-targeted proteins are released from the ER during the early UPR.

(A) Localization of the translation of mRNAs encoding ER-targeted (red) proteins and cytosolic (blue) proteins over the treatment time course. Also indicated in the level of eIF2α phosphorylation (black) as quantified from Fig. 1A. (B) Changes in the mRNA concentration, translational efficiency, and total translation for ER-targeted proteins over time. (C) Density plot of $\log_2(R_t/R_0)$ separated by compartment and protein type. Red line represents the median value of each set. More intense green points represent more genes. (D) Trajectories of the translation of all mRNAs encoding ER-targeted proteins over the treatment timecourse, where blue indicates an increased ER localization, red decreased, and values that change more are more opaque. (E) Same for mRNAs encoding ER-targeted proteins. (F) Relationships between the change in localization of translation and the change in localization of mRNAs encoding ER-targeted proteins for each timepoint transition. Best fit lines are also shown. (G) Selected mRNAs encoding ER-targeted proteins whose translation is retained or recruited to the ER following induction of stress.

Another strategy that cells use to decrease the ER protein folding load in the UPR has been described: the degradation of ER-associated mRNAs by the ER membrane nuclease IRE1α (RIDD) (Gaddam et al., 2013; Hollien and Weissman, 2006). To test whether RIDD was active in our experimental system, we plotted the $\log_2$ fold change in transcript level against the localization of that mRNA. There was a modest but statistically significant relationship between ER localization and change in transcript levels, where a 10% increase in ER localization corresponded to a 3% decrease in mRNA abundance at the peak of the effect, with a great deal of variability between transcripts (Fig. Figure 18C,D). This demonstrates that RIDD, while detectable, played a relatively small role in our experimental system.

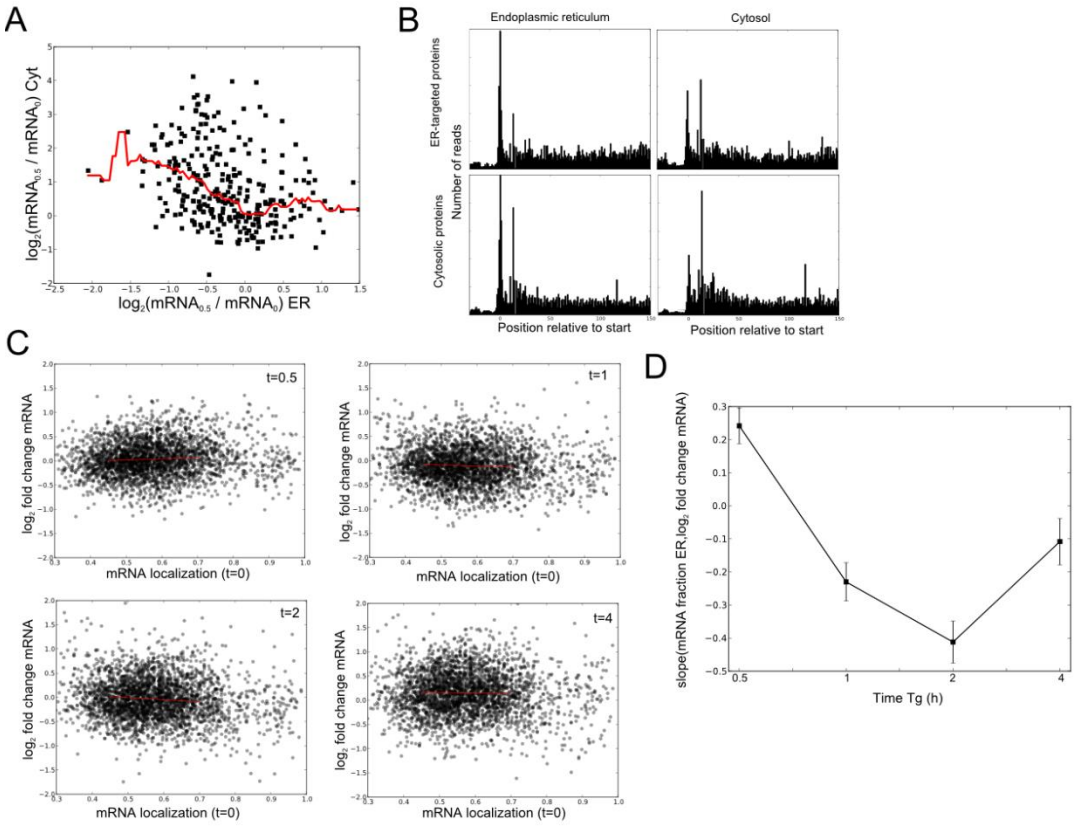


Figure 18: Exchange of mRNAs between the cytosol and ER.

(A) For all mRNAs encoding ER-targeted proteins, the relationship between the log fold change in the cytosol and in the ER after 0.5 h Tg is plotted. Red line is a moving average. The negative correlation indicates that, in general, a reduction in one compartment gives rise to a corresponding increase in the other. There are significant exceptions, which likely represent mRNAs that are transcriptionally induced or degraded. (B) The distribution of ribosome binding for all mRNAs encoding either cytosolic or ER-targeted proteins in the cytosolic or ER cell fractions. Each x axis is generated relative to the start codon. (C) The prominence of RIDD is characterized by plotting the log fold change in RNA levels relative to resting cells as a function of mRNA localization in resting cells for each time point. (D) The slopes of the best fit lines from (C) are plotted over time. Error bars represent standard deviation over experimental replicates.

Despite the large-scale recompartmentalization of mRNAs to the cytosol, a small subset

of mRNAs encoding ER-targeted proteins gained or retained ER localization. Those

mRNAs that remained on the ER during this early restructuring of the transcriptome likely contribute either to the decrease in protein folding load or increase in protein folding capacity of the ER – generally, functions essential to the recovery of homeostasis in the ER. Several ER-retained mRNAs encoded proteins that degrade or remove proteins from the ER. Genes involved in proteolysis, exocytosis, and ER-associated degradation were all represented (Figure 17G). Perhaps most outstanding was the near-uniform retention of the syntaxins and other mRNAs whose protein products are involved in protein export from the ER by vesiculation. The GO term "SNARE complex" (GO:0005484) increased its localization to the ER by 1.4%, compared to a mean of all GOs for ER-targeted proteins of -35%. In principle, the sequestration of unfolded proteins into vesicles could provide a potent means to decrease protein folding load, and there is precedent for such activity in bacterial unfolded protein stress (Schwechheimer and Kuehn, 2013). Conversely, mRNAs encoding ER lumenal chaperones were released from the ER similarly to most mRNAs encoding ER-targeted proteins; Grp94 (-43%), BiP (-38%), and Calreticulin (-44%) were all efficiently released. This indicates that the early portion of the UPR focuses primarily on reduction in protein folding load rather than increase in protein folding capacity. Also retained on the ER was Scap, which promotes sterol synthesis, perhaps to allow for expansion of ER volume. Together, these features further define the approaches used by cells to immediately adapt to unfolded proteins in

the ER while demonstrating the specificity of the recompartmentalization of the translation.

4.4 Enhanced translation of cytosolic proteins occurs on the ER

Finding a major recompartmentalization of mRNAs encoding ER-targeted proteins, we next investigated the fate of mRNAs encoding cytosolic proteins in the UPR. We noted a significant positive correlation ($r=0.40$; p-value<0.001) between the change in total translation after 0.5 h treatment and the fraction of translation that occurs on the ER (Figure 19A). Here, a 10% increase in translation's ER localization corresponded to a 19% increase in total translation. This relationship indicates that the ER, having been depleted of a fraction of its associated mRNAs, serves as a privileged platform for accommodating those transcripts whose translation is to be enhanced in the UPR. The magnitude of this correlation peaked at 0.5 h, then fell as the cell redeveloped homeostasis and mRNAs encoding ER-targeted proteins returned to the ER (Figure 19B). No such correlation was apparent, however, between the localization of the mRNAs themselves and the change in their translation. The divergence between translation and mRNA localization demonstrates that the changes in translation of cytosolic proteins is a result not of mRNA relocalization, but rather of those mRNAs that are already on the ER experiencing enhanced translation in that compartment. This effect may be a result of translational machinery (ribosomes, tRNA synthetases, etc), left idle by the release of mRNAs encoding ER-targeted proteins, being repurposed to

translate cytosolic proteins.

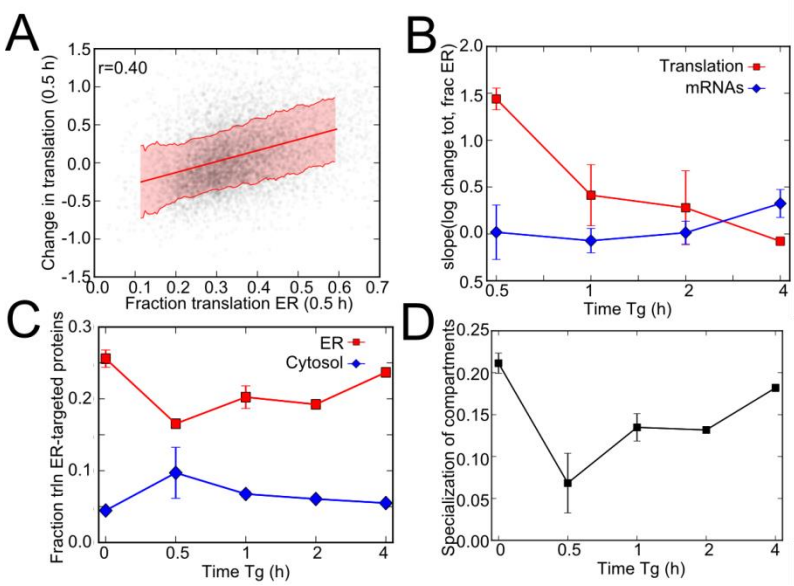


Figure 19: The translation of cytosolic proteins moves to ER-bound ribosomes.

(A) For all cytosolic proteins, the log change in translation after 0.5 h Tg is plotted against the ER enrichment of that translation at 0.5 h. Red line is a linear best fit and shaded red area represents ± SD. (B) The slopes of the linear best fit as in (A) is plotted over treatment time for translation and mRNA. (C) The fraction of ribosomes engaged in the translation of ER-targed proteins is plotted for each cellular compartment over the treatment time course. (D) Specialization for ER-targeted protein synthesis of the ER and cytosolic compartment over time, as described in Methods, where 0 represents no divergence and 1 is complete divergence.

The large-scale recompartmentalization of translation for mRNAs encoding both cytosolic and ER-targeted proteins resulted in significant changes in the composition of translation in each compartment. For the ER, 26% of ribosomes are engaged in the synthesis of ER-targeted proteins in untreated cells (Figure 19C). After 0.5 h treatment, this number dropped to 16% before slowly recovering over the remainder of the time course. The inverse relationship was apparent in the cytosol, where there was little

translation of ER-targeted proteins in resting cells, but a greater amount at 0.5 h treatment. Together, these recompartmentalizations amounted to a de-specialization of the two primary compartments for translation (Figure 19D). While the ER contained a much greater proportion of translation of ER-targeted proteins than the cytosol in untreated cells, the combined changes in translation upon Tg treatment made the two compartments barely distinguishable. This change is likely adaptive – with the translation of more ER-targeted proteins harmful to the restoration of protein folding homeostasis in the ER, it becomes beneficial to repurpose the ER translational apparatus for general protein synthesis.

4.5 Rapid kinetics of translational reorganization and recovery

To gain further insight into the kinetics of stress-induced mRNA re-compartmentalization, we used an alternative stressor, DTT, which interferes with disulfide bond formation in the ER . In these experiments, we treated MEFs for a period from 2-30 min, then eliminated the stressor by replacing with fresh media lacking DTT for a subsequent time course. Following treatment, total translational activity, measured by [^{35}S]Met/Cyt incorporation, dropped sharply initially, then more gradually until it plateaued at ~30min (Figure 20A). Upon release of the stress, translation immediately began to recover, although it did so fairly gradually (~2% per min). The phosphorylation of eIF2α is induced more slowly, but recovers more rapidly (Figure 20B). To assess the kinetics of translation and mRNA relocalization, we treated cell for 30 min with DTT,

then allowed them to recover for 10 min or 20 min, then analyzed each timepoint by ribosome profiling. Ribosomes were modestly released from the ER upon treatment, then began to return within 10 min (Figure 20C). The release of translation of ER-targeted proteins was recapitulated after 30 min DTT treatment (Figure 20D). Following DTT washout, mRNA localization recovered rapidly at a transcriptome scale, with most of the recovery complete after 10 min and continuing to recover at 20 min. The translational response to DTT also resembled that to Tg (Figure 21A). Together, these results demonstrate that the release and retrieval of mRNAs encoding ER-targeted proteins occurs within the first minutes of stress induction and recovery in a manner correlated with eIF2α phosphorylation.

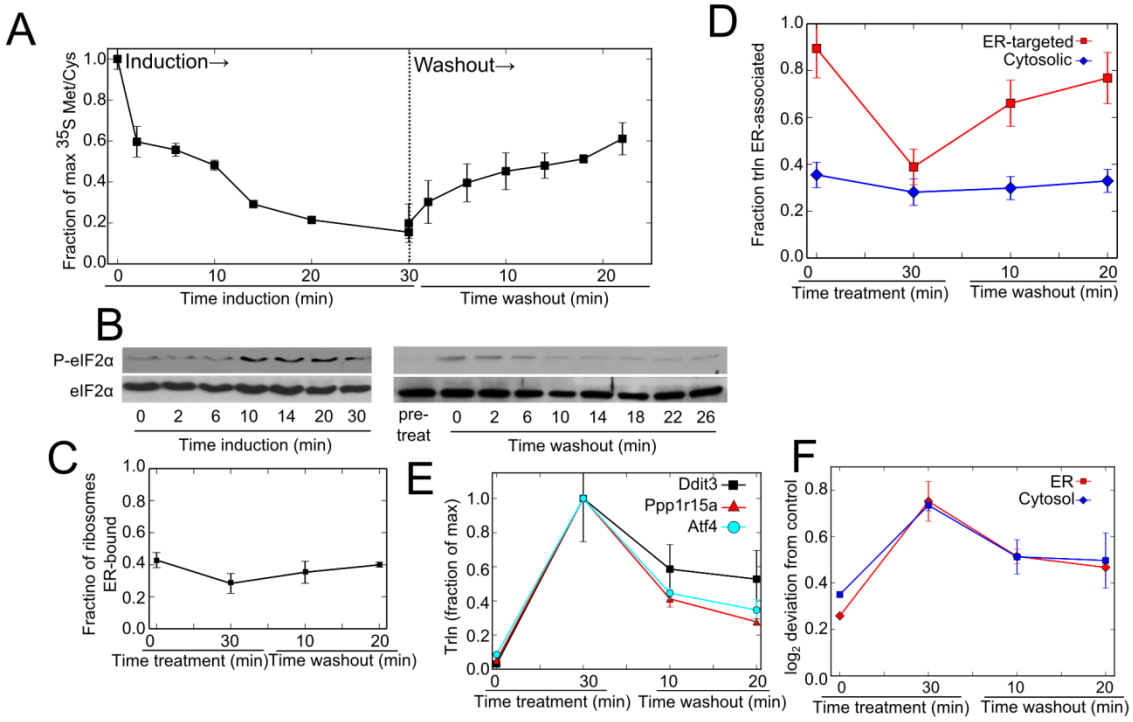
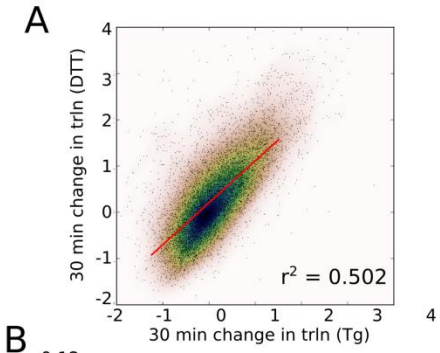


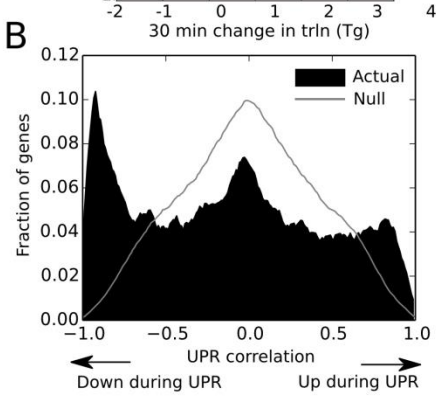
Figure 20: Rapid kinetics of removal and retrieval of mRNAs.

(A) Translational activity as measured by [^{35}S]Met/Cys incorporation over a time course of treatment with 1 mM DTT. At 30 min, DTT-containing media is replaced with fresh media and allowed to recovery over a time course. (B) Phosphorylation kinetics as assessed by western blot during DTT treatment and washout. (C) Fraction of ribosomes associated with the ER during DTT treatment as washout. (D) Localization of the translation of ER-targeted proteins and cytosolic proteins as assessed by ribosome profiling during the induction are recovery from DTT treatment. (E) Patterns of induction and recovery of total translation for canonical targets for UPR translational regulation. (F) Changes in gene-level translation for cytosolic and ER-associated ribosomes in UPR inducation and recovery. The median value of the absolute $\log_2$ deviation in translation in calculated in each compartment, with error bars representing standard error between replicates. At to 0 timepoint, points represent the deviation in experimental replicates for Control samples.

Also notable was the rapid recovery of gene-level translational efficiency upon the release of stress. Canonical targets for translational up-regulation in the UPR had significantly enhanced translation after DTT treatment, and this enhancement dropped off rapidly after washout of DTT (Figure 20E). This pattern was also apparent at a transcriptome scale. Upon induction of stress with DTT, both ER and cytosolic translational efficiency deviated prominently from experimental noise (Fig. 4F). Following DTT washout, each compartment rapidly began to return to the translational state of untreated cells, with about half of the deviation absent after 10 min and continuing to decrease after 20 min. This deviation had similar kinetics in each compartment. Additionally, these short timepoints allowed for the identification of novel targets of translational regulation in the UPR – in this time scale, translation will be the dominant factor in gene regulation and few complicating variables have time to arise. We scored genes based on how closely they follow a pattern of up-regulation in

the UPR and rapid recovery following release of stress (Figure 21B). Rapidly responsive genes clustered in categories previously discussed, where ER-targeted protein are down-regulated and several regulatory functions are up-regulated (Figure 21C). Curiously, although presence of upsteam open reading frames have been characterized as leading to enhanced translation in the UPR for several genes (Lu et al., 2004), we found weak correlation between these gene elements and translational changes in the UPR (Fig. Figure 21D,E). These results indicate a rapid, stress-responsive translational regulatory system that operates by a mechanism largely independent of upsteam open reading frames.





C

GO	Description	Avg. corr.	pval
	Up in UPR		
GO:0031369	translation initiation factor binding	0.478	0.0002
GO:0016581	NuRD complex	0.419	0.0008
GO:0033613	activating transcription factor binding	0.328	0.0068
GO:0005720	nuclear heterochromatin	0.307	0.0152
GO:0006479	protein methylation	0.284	0.0096
	Down in UPR		
GO:0031225	anchored to membrane	-0.851	<0.0002
GO:0005788	endoplasmic reticulum lumen	-0.785	<0.0002
GO:0001558	regulation of cell growth	-0.636	<0.0002
GO:0005125	cytokine activity	-0.550	0.0002
GO:0006935	chemotaxis	-0.474	0.007

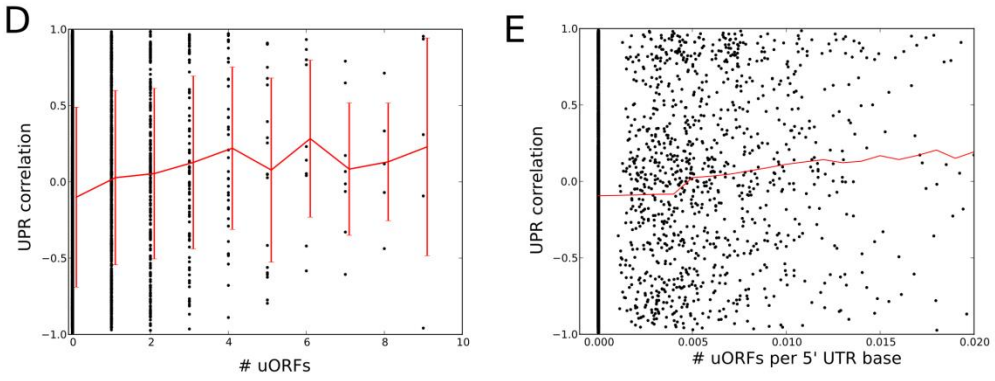


Figure 21: Identification of mRNAs whose translation is rapidly responsive to the UPR.

(A) Translational changes in Tg are compared to those in Tg. The correlation of total translation over the DTT timecourse (no treatment, 30 min DTT, 10 min recovery, 20 min recovery) and a pattern of (0, 1, 0, 0) was calculated for each gene. In (B), a histogram for all genes is shown along with a null distribution that was generated by drawing random values for translational levels is shown. (C) The enrichment of positive or negative correlations in gene ontologies was calculated by bootstrapping. Selected ontologies are shown. (D) Relationship between number of upstream open reading frames and

translation response in the UPR. Red line indicates the average value; error bars indicate standard deviation (E) Relationship between density of upstream open reading frames in the 5′ UTR. Red line is a moving average.

4.6 Compartmental regulation of translation by GADD34 and CReP

Having established compartment-specific regulation of translation, we asked how this process might be regulated. We focused on GADD34 and CReP, each of which has been identified as promoting the eIF2α phosphatase activity of PP1 (Connor et al., 2001; Jousse et al., 2003). CReP is generally characterized as having constitutive activity in maintaining low eIF2α phosphorylation status, while GADD34 is primarily inducible (Jousse et al., 2003). We were, however, able to detect GADD34 translation in untreated cells, indicating that it could have a role in maintaining basal eIF2α phosphorylation status (Figure 22A). CReP is partitioned to the ER, while GADD34 is largely cytosolic (Zhou et al., 2011), making them promising candidates for compartmental regulation of translation. Using MEFs with homozygous deletions in either gene (Fig. 5A), we analyzed the translational response to Tg as above to determine what roles they play in unfolded protein response and recovery. Curiously, in CReP$^{-/-}$ cells, GADD34 translation was elevated in untreated cells, perhaps indicating a compensatory response to ongoing stress in this mutant (Figure 22A). GADD34$^{-/-}$ cells failed to recover protein synthesis levels during Tg stress and continued to accumulate phospho-eIF2α over the entire timecourse after starting from a higher basal level (Figure 22A,B), as demonstrated previously (Brush et al., 2003). These findings indicate that basal GADD34

expression is required for the reversal of eIF2α phosphorylation, and by extension, protein translation. Surprisingly, CReP$^{-/-}$ cells were indistinguishable from WT in both protein synthesis and eIF2α phosphorylation, indicating that under these experimental conditions, CReP activity does not contribute significantly to the regulation of eIF2a activity during the immediate, pre-transcriptional response phase of the UPR (Figure 22B).

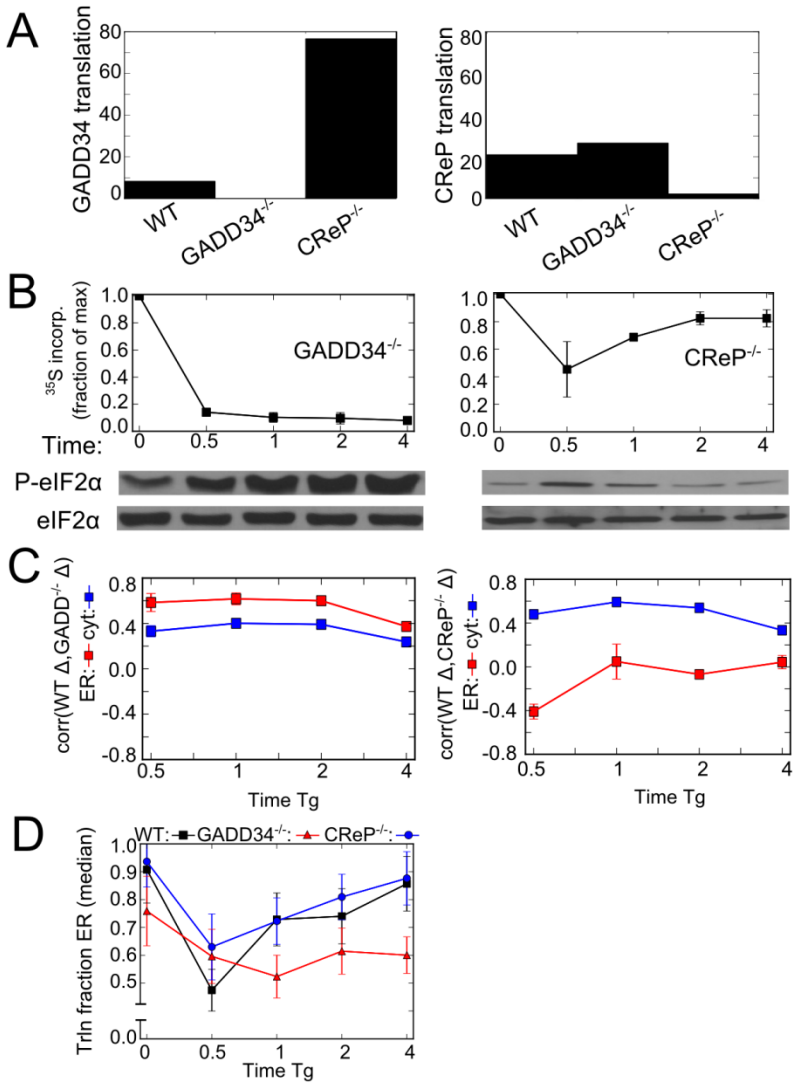


Figure 22: GADD34 and CReP are required for the UPR translational program in distinct cellular compartments.

A) Levels of GADD34 and CReP translation in WT, GADD34-/-, or CRep-/- cells as detected by ribosome profiling. (B) Translation rates measured by [^{35}S]Met/Cys incorporation following Tg treatment for GADD34$^{-/-}$ (L) and CReP$^{-/-}$ (R) cells. Below, western blots against eIF2α and P-eIF2α over the same time points. (C) Correlations between translational response in WT cells and translational response in mutant cells, where translational response is $\log_2(R_{time}/R_0)$ (D) Median fraction of translation on the ER for ER-targeted proteins over the treatment timecourse for WT, CReP$^{-/-}$, and GADD34$^{-/-}$ cells. Error bars represent standard deviation of the localization distribution.

To test whether either gene knockout changes the compartmental regulation of translation, we analyzed translation in the cytosol and ER in each knockout cell line by ribosome profiling. We then compared the UPR-induced changes in translation that occur in each knockout to those that occur in WT cells by calculating the correlation coefficient between the $\log_2$ change in translation in WT compared to the $\log_2$ change in translation in each mutant at each time point (Figure 22C). In GADD34$^{-/-}$ cells, the translational response on the ER well resembled that in WT cells. However, the translational response to Tg in the cytosol was almost completely reprogrammed, indicating that GADD34 is necessary for the cytosolic portion of the translational UPR. In marked contrast, CReP$^{-/-}$ cells resembled WT cell in their cytosolic response, while on the ER, they have zero or negative correlation, indicating the CReP activity is focused on the regulation of ER translation. This change occurs despite CReP having no apparent effect on eIF2α phosphorylation status (Figure 22B), leaving its mechanism of action mysterious. We next investigated whether each mutant cell type recapitulates the recompartmentalization of mRNAs encoding ER-targeted proteins to the cytosol that

was observed in WT cells, finding that they differ in distinct ways (Figure 22D). For GADD34$^{-/-}$ cells, this class of mRNAs is poorly ER-enriched even before treatment, likely reflecting the chronically high eIF2α-P levels, then falls even further upon chronic stress and fails to recover. In CReP$^{-/-}$ cells, the magnitude of recomparmentaliztion is reduced by nearly half relative to WT, indicating that CReP is a critical contributor to the UPR-stimulated compartmental reorganization of translation. Together, these findings demonstrate that the compartmental regulation of translation is modulated by proteins that are localized to and have activity towards particular fractions of the cell.

4.7 Perspective: translational compartmentalization in regulatory networks

The UPR develops in response to a highly compartmentalized stress in the cell and couples proteostasis in the ER lumen to global translational regulation of proteome expression. Here, we have demonstrated that the subcellular architecture of translation is remodeled in a manner that is uniquely well suited for this stimulus. In order to stem the influx of unfolded proteins into the ER, the mRNAs that encode ER-targeted proteins are selectively released from the ER membrane. Simultaneously, the translation of mRNAs encoding cytosolic proteins – which mostly remain on the ER – is relatively enhanced, likely taking advantage of the translational machinery left behind by the mRNAs that have departed. This recompartmentaliztion occurs rapidly and recovers at a similar rate after elimination of the stress. These findings define mRNA and ribosome

localization to the ER as a new variable in translational regulation that is relevant to virtually all transcripts and is dynamic in response to stimuli.

4.7.1 Dynamic association of polyribosomes with the ER

When considering the means by which polyribosomes can adhere to the ER membrane, it is useful to consider three general classes of interactions, each of which is likely conferred by several separate molecular mechanisms (Figure 23A). First, nascent protein chains can provide affinity for the ER (Kalies et al., 1994). Second, ribosomes can associate with the ER independently of translation and can initiate translation while maintaining ER association (Borgese et al., 1973; Hortsch et al., 1986; Savitz and Meyer, 1990; Seiser and Nicchitta, 2000). Finally, mRNAs themselves can retain ER association independently of ribosomes (Chen et al., 2011). Each of these interactions must be absent or disrupted to allow for release of a polyribosome. Alternatively, a ribosome may dissociate from an mRNA, allowing for release of the mRNA while retaining ribosome affinity. Within this framework, we will now consider how changes in each of these variables could give rise to the observed selective release of mRNAs encoding ER-targeted proteins.

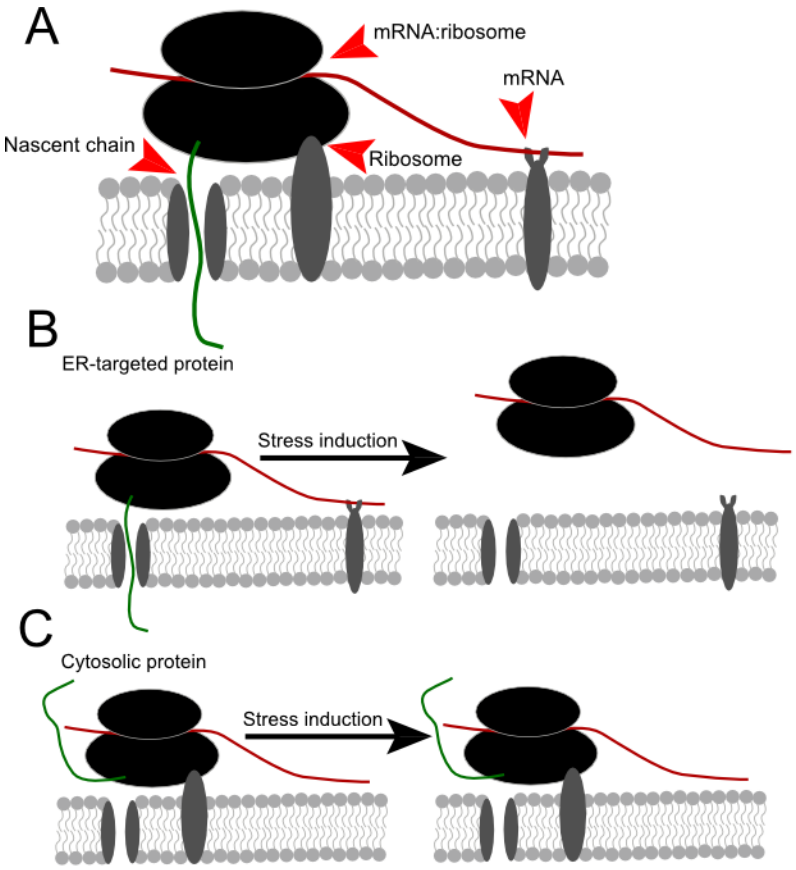


Figure 23: Models for dynamic mRNA localization to the ER.

(A) Points at which polyribosomes can associate with the ER are indicated by red arrows (red line is mRNA, green is nascent protein, black ellipses are ribosomal subunits). In addition to modification mRNA, ribosome, and nascent chain interactions with the ER, an mRNA may be released from the ER in the case where the mRNA dissociates from the ribosomes while the ribosomes itself retains affinity. (B) Model of the changes that occur for a polyribosome engaged in the synthesis of an ER-targeted protein whose ribosomes are not independently associated with the ER in ER stress. Here, ER:nascent chain and ER:mRNA interactions are disrupted, allowing for the release of the polyribosome. (C) Same as (B) for a polyribosome synthesizing a cytosolic protein whose ribosomes are independently associated with the ER. Having no nascent chain or mRNA interaction with the ER, the polyribosome is retained on the ER due to the ongoing interaction between the ribosome and the ER.

In normal conditions, polyribosomes synthesizing ER-targeted proteins are generally anchored to the ER by two mechanisms: by their nascent protein chain and by their mRNAs. The ribosomes themselves may or may not have independent affinity for the ER. Polyribosomes synthesizing cytosolic proteins, in contrast, do not have affinity conferred by their nascent proteins and their mRNAs are not tethered to the ER. These polyribosomes, therefore, must be anchored to the ER by ribosomes that have their own independent ER affinity (Borgese et al., 1973). It follows that selective release of mRNAs encoding ER-targeted proteins could be achieved by disrupting ER:nascent chain and ER:mRNA interactions while leaving some ER:ribosome interactions intact (Figure 23B). mRNAs that have at least one ribosome that has ER affinity would retain its ER association, allowing for the retention of mRNAs encoding cytosolic proteins and a fraction of mRNAs encoding ER-targeted proteins (Figure 23C). Specific mRNAs, such as those identified that are important for homeostasis recovery in the ER lumen, could also be retained by association with specific mRNA-binding proteins that are not disrupted in ER stress. Together, these variables provide a framework for understanding the dynamics of polyribosome association with the ER. Indeed, some aspects of this system have already been described. ER:nascent chain interactions have been shown to be disrupted in the UPR in a system where the proteasome degrades nascent ER proteins cotranslationally (Oyadomari et al., 2006). A diversity of ribosome receptors has been identified, providing ample room for distinct responses in the UPR (Kalies et al.,

1994; Kreibich et al., 1978; Savitz and Meyer, 1990). The study of mRNA tethering to the ER, however, is young, and identification of mRNA receptors and their dynamics requires further study.

4.7.2 Points of regulation for mRNA compartmentalization

A critical component of the translational arm of the UPR is the phosphorylation of eIF2α. In addition to conferring a general down-regulation of translational activity, P-eIF2α is well correlated with mRNA relocalization over short and long timecourses and in several mutant cell lines. It is tempting to speculate that eIF2α phosphorylation is responsible, directly or indirectly, for the release of specific mRNAs from the ER. Important players in this system are GADD34 and CReP. While each has been characterized as directing phosphatase activity towards eIF2α, we find dramatically divergent responses to their absence. In GADD34 knockout cells, eIF2α phosphorylation continues to build over time instead of recovering, while CReP knockout cells have eIF2α phosphorylation patterns that are indistinguishable from WT cells. This is surprising in two ways. First, GADD34 has been generally reported to be solely inducible and undetectable in resting cells (Harding et al., 2009; Kojima et al., 2003). However, we find that its gene knockout has appreciable effects on mRNA localization in the absence of stress induction and that translation of GADD34 is detectable in resting cells (Fig. 5A, 5D). This expands the scope of GADD34 function to include maintenance of steady-state translational homeostasis in addition to stress response. Secondly, the

absence of a detectable effect of CReP on the kinetics of eIF2α phosphorylation suggests additional or alternative functions for CReP in the translational response to UPR activation. While CReP knockout cells had eIF2α phosphorylation profile that was indistinguishable from WT, they had a significant impact on the cell's response to stress, particularly on the ER. Given that CReP knockout hinders mRNA release from the ER, it is plausible that CReP, in an as yet unknown manner, modulates ER-mRNA interactions. The compartmentalization of these the proteins' activity is also of note: CReP is associated with the ER and primarily modifies ER translation, while GADD34 is mostly cytosolic and modifies cytosolic translation. This system of separate regulatory factors in each compartment could form the basis for a compartmentalized regulation of translation. Together, these findings demonstrate that the substrates, dynamics, and downstream effects of GADD34 and CReP are likely far more complex than previously appreciated.

While the UPR is a prominent example of a stimulus leading to eIF2α phosphorylation, there are many physiological inputs that result in that same output. Amino acid starvation, viral infection, and heavy metal exposure can all stimulate eIF2α phosphorylation by various kinases (Samuel, 1993). This raises the possibility that the sort of response we observe here, with specific mRNAs being recompartmentalized, may in fact be generalizable to a number of cell stresses.

Methods

Cell culture and treatment

Cells were cultured in DMEM + 10% FBS at 37C in 5% CO_2 and were harvested at ~80% confluence. Cell lines used were: WT MEFs, GADD34$^{-/-}$ MEFs, and CReP$^{-/-}$ MEFs (Harding et al., 2009), each of which was SV-40 immortalized. Cell fractionation was carried out according to (Jagannathan et al., 2011). Cells were treated with 180 μM cycloheximide, washed with cold PBS, then plasma membrane permeablized in 110 mM KOAc, 25 mM K-HEPES pH 7.5, 15 mM $MgCl_2$, 4 mM $CaCl_2$, 0.03% digitonin. The ER was solubilized in 200 mM KOAc, 25 mM K-HEPES pH 7.5, 15 mM $MgCl_2$, 4 mM $CaCl_2$, 2% dodecyl-maltoside. Cells were treated with 1 μM thapsigargin (Calbiochem) or 1 mM DTT from a 1 mM or 1 M stock.

Radioactive labeling and ribosome counting

Cells were starved of Met/Cys for 30 min. Cells then were pulsed with 50 μCi/mL [^{35}S]Met/Cys and for 5 min and treated with 180 μM cycloheximide to halt labeling. Cells were lysed in 1% CHAPSO, 200 mM KOAC, 15 mM KHEPES pH 7.2. Trichloroacetic acid was added to 10% and incubated for 20 min on ice to precipitate protein, then passed through cellulose filters and rinsed thoroughly with 10% TCA + 10 mM Met and EtOH. Filters were then evaluated by liquid scintillation counting.

Ribosome counting

Cells were fractionated and ribosomes pelleted through a 500 mM sucrose cushion (90,000 RPM for 30 min, TLA 100.2 rotor). The ribosome pellet was resuspended and RNA purified by GT/phenol extraction. The concentration of ribosomes was then approximated by recording absorbance at 260 nm using a U-2000 spectrophotometer (Hitachi).

Western blotting

Treated cells were lysed in 1% CHAPSO, 200 mM KOAC, 15 mM KHEPES pH 7.2, precipitated with trichloroacetic acid, separated by SDS-PAGE, transferred to nitrocellulose, and were blotted according to manufacturer's instructions. All blots were loaded as cell equivalents.

Ribosome profiling, library construction, and sequencing

Ribosome profiling was performed essentially according to (Reid and Nicchitta, 2012). Cell lysates representing cell fractions of one 10 cm dish were adjusted to 100 mM KOAc by dilution and treated with 10 ng/μL micrococcal nuclease (Sigma-Aldrich) for 30 min at 37 C. Ribosomes were isolated by centrifugation over a 500 μL 500 mM sucrose cushion in a TLA 100.3 rotor (90,000 RPM; 36 min). Ribosome pellets were resuspended in 400 μL 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% N-lauryl sarcosine, 5 mM EDTA and RNA purified by phenol/chloroform extraction (Stephens et al., 2008). After precipitation, the RNA was prepared for library construction by resuspending in

10 μL 1U/μL T4 polynucleotide kinase (New England Biolabs), 100 μM ATP and incubated at 37 C for 1 h. The reaction was then diluted in 10 μL formamide loading buffer (80% formamide, 10 mM EDTA, 1 mg/mL xylenol blue, 1 mg/mL bromophenol blue). Ribosome footprints were loaded onto a gel composed of 15% acrylamide, 8M urea, and 2 mM EDTA, 89 mM Tris-borate pH 8.3. RNA was run at 400 V until just before the xylonol blue ran off the gel. Ribosome footprints, which ran just above a 35-nt DNA oligonucleotide, was visualized by SyBr Gold (Invitrogen) and extracted by crushing and incubation in 400 μL 400 mM KOAc for 2 h while shaking. The extracted RNA was then passed through a SpinX cellulose filter to remove acrylamide and precipitated by addition 1 mL EtOH. Libraries were prepared using the NEBNext Small RNA kit (New England Biolabs) and amplified using 15 cycles of PCR. In parallel, a fraction of each lysate was used for mRNA sequencing. rRNAs were depleted by RiboMinus (Life Technologies) and libraries prepared with the Illumina TruSeq RNA kit. Libraries were bar coded and sequenced using the Illumina HiSeq 2500, acquiring 50 bp reads.

Mapping and quantification of reads

Reads were mapped to an index of mouse RefSeq mRNAs (longest coding sequence for each mRNA only) using Bowtie 1.0 using a 20 nt seed region and allowing for one mismatch the two best reported locations for each read (Langmead et al., 2009). The ribosome density, R, is calculated based on RPKM (mapped reads per kilobase coding

sequence per million total reads). mRNA reads were mapped according to the same protocol. All libraries were normalized a factor representing the fraction of ribosomes in the relevant compartment and time point.

Data analysis

Data were analyzed using a series of custom Python scripts and plotted using Matplotlib. Unless otherwise specified, error bars represent the standard deviation between experimental replicates. The position of each ribosome was defined at the beginning of each read +13 nt. For purposes of analyzing mRNAs encoding ER-target proteins, we defined ER-targeted proteins as those that contain either a signal sequence or transmembrane domain as determined by TMHMM (Krogh et al., 2001).
Gene ontology analyses was performed by calculating the mean of all components of that each ontology. P-values were calculated by randomly shuffling gene values, then re-calculating each mean. Upstream open reading frames were defined as those regions in 5′ untranslated regions that contain an AUG with an in-frame stop codon.

5. Perspectives

These studies have taken an unbiased recording of the localization of mRNAs to the ER in tissue culture cells, analyzed how translation is distributed between the cytosol and ER, and identified a role for dynamic mRNA localization in the cellular response to unfolded proteins. These finding expand the role for ER-bound ribosomes from exclusively and constitutively synthesizing membrane and secretory proteins to a role

that is central to the synthesis of proteins throughout the cell and that can be reshaped in order to suit the needs of the cell.

These studies raise an enormous range of questions regarding the mechanisms that give rise to the observations we have made and the ways that cells (and pathogens) may take use them to further their own means. I will speculate briefly about each of these.

There are several classes of mechanisms of interactions that may be relevant to this field of study. Perhaps most obvious is the tethering of mRNAs to the ER. There are several lines of evidence in this work that point towards multiple categories of mRNA tethering mechanisms on the ER: mRNAs encoding a subset of ER-targeted proteins are selectively retained after disruption of ribosome:ER interactions and mRNAs encoding ER-targeted proteins are selectively released from the ER during ER stress. This indicates that there are at least three sorts of mRNA interactions with the ER: those for mRNAs encoding cytosolic proteins, those for ribosome-dependent ER interactions of mRNAs encoding ER-targeted proteins, and those for ribosome-independent ER interactions of mRNAs encoding ER-targeted proteins. These three classes of mechanisms of ER association have been a subject of ongoing study in the Nicchitta laboratory, and we have made some compelling observations that each group of mRNAs rely on dramatically divergent mechanisms. I will not, however, discuss this work here.

Other factors – ribosomes, elongation and initiation factors, and tRNAs, for example – could be recruited to the ER as additional layers of compartmental translational regulation. Several of these components have in fact been found to have their own independent affinity for the ER. Ribosome receptors were a very active field of study for many years, and many were identified with varying degrees of confidence. Although it is difficult to conclude with certainty that any one of these factors is involved in ribosome binding to the ER, it is certain that ribosome binding to the ER is far more complex than the Sec61:ribosome interaction that is most well known, especially given that Sec61 is present at only 1-2 copies per 100 ribosomes in yeast cells (Gorlich et al., 1992). Some tRNA synthtases can also associate with the ER (Dang et al., 1983). Together, the co-localization of these molecules to the ER could comprise an independent compartment within the cytosol that is competent for translation without contribution from free cytosolic factors. This concentration of factors, in addition to presenting potential regulatory opportunities, may provide a significant kinetic advantage: by concentrating the substrates and enzymes in protein synthesis, particularly on a two-dimensional plane, the ER could serve as a favored site for translational initiation or elongation (Hiemenz and Rajagopalan, 1997).

Many of the findings in this work raise questions regarding the role of SRP in mRNA localization and translational localization. In early proposals for SRP function, it plays a role in mRNA localization, ribosome targeting, and protein translocation. The

observation that mRNAs that lack a signal sequence or transmembrane domain are translated on the ER makes it implausible that SRP is *necessary* mRNA localization or translational targeting. Several studies have used knockdowns or knockouts of SRP components to shed light on which component(s) of the model proposed by Blobel are reflected in cells. *In Saccharomyces cerevisiae*, SRP depletion resulted modest defects in protein translocation, but cells continued to be viable (Hann and Walter, 1991). A later study in *Trypanosoma brucei* found that loss of SRP function resulted in disruption of membrane protein topology but no effect on proteins that encoded only a signal sequence (Lustig et al., 2007). Furthermore, mRNAs retain ER association even after siRNA-mediated knockdown of an SRP constituent protein (Pyhtila et al., 2008). Together, these observations point towards a much narrower role for SRP than initially proposed by Blobel. SRP is generally dispensable for mRNA localization, translational localization, and protein targeting, but seems to play an important role in ensuring that membrane and secretory proteins are properly inserted into the ER lumen. This refined role as a membrane and secretory protein chaperone is reinforced by its binding site on the ribosome, which is redundant with the co-translational protein chaperone NAC (Wiedmann et al., 1994).

With the complexity and intricacy of translational compartmentalization becoming clear, we can now begin identifying mechanisms for a whole host of functionalities regarding translation on the ER. One fundamental property that remains

to be explored is the means by which ribosomes associate with the ER. A substantial number of ER proteins have been identified as having ribosome binding activity. Invoking heterogeneous ribosome binding could allow for a number of exciting functionalities – for example, a ribosome bound to ribophorin could be specialized for the translation of one subset of mRNAs while a ribosome bound to Sec61 could be specialized to translate another. Furthermore, there may be a population of ribosomes that is not directly bound to the ER at all, but instead is tethered *via* an mRNA that it is translating. Some evidence for this occurring is apparent in (that EM study). All told, divergent ribosome binding activity could have broad consequences for polysome functionality.

The diversity of mRNA binding activity that I have begun to describe here is certainly conferred by a broad array of mechanisms. As these mechanisms begin to be described, it seems likely that they will defy the canonical mRNA localization mechanisms that are well studied, such as actin mRNA localization to the leading edge of migrating cells (Sundell and Singer, 1990) or *oskar* and *nanos* in the *Drosophila* embryo (Kugler and Lasko, 2009), where a particular mRNA binding protein binds a particular region on the mRNA and is trafficked or anchored to the locale of choice. I argue this for two reasons. First is simply that there are so many more mRNAs present on the ER relative to other mRNA localizations. In, for example, neurons, the target site is relatively small and there are relatively few mRNAs that are localized to that site

(Martin and Ephrussi, 2009). This system would require these receptor:ligand interactions to have quite low k_{DS} and quite high specificity relative to other mRNAs. In contrast, the ER in most cells is quite abundant, there are many abundant ER membrane components that could plausibly serve to bind mRNAs, and virtually all mRNAs bind to the ER to some degree. In this system, mRNA receptors (likely proteins, but plausibly other molecules) could have relatively high k_{DS} and relatively low specificity. Such a system could involve a wide range of mechanisms that have not yet been considered, much less identified. Regardless of the mechanism, one thing has become clear over the course of these studies: there exists no obvious primary mRNA sequence signature that corresponds to ER association. Despite rigorous searching using numerous computational approaches, there are no strong candidates – the landscape of defining affinity is likely to be far more complex (Polyansky et al., 2013).

With mRNAs, ribosomes, and potentially other molecules relevant to translation (initiation and elongation factors, mRNA binding proteins, chaperones, tRNA synthetases, and so on), we can begin to imagine a system wherein translation function as a enzymatic process that occurs mostly with the two-dimensional plane of the ER membrane. This restriction of an enzymatic process into two dimensions raises a number of questions that normally need not be addressed in enzymology. One prominent impact is that by of diffusing in two dimensions instead of three, substrates and enzymes would experience an increased observed concentration, thus enhancing

rates of initiation and elongation (Berry, 2002; Castronovo et al., 2011; Gosele and Huntley, 1975). This concentration of reactants could be a primary rationale for synthesizing even many cytosolic proteins on the ER: concentration of reactants on the surface of the ER would require fewer components be present in order to achieve the same rate. Some aspects of this two-dimensionality, however, seemingly work against efficient translation on the ER. Perhaps most interesting is the problem of multiple components associating with the ER. As an extreme example, suppose that an mRNA is directly associated with the ER, as are all of its associated ribosomes. This would impose an additional energetic cost of ribosomal procession: in addition to moving along the mRNA, the ribosome's ER tether would have to be moved relative to the mRNA's ER tether. Furthermore, diffusion in a lipid bilayer can be quite slow relative to water (Peters and Cherry, 1982). This may counteract some of the advantages of the relative enhancement of concentrations. Regardless, collapsing a enzymatic process as complex as protein synthesis onto a two-dimensional plane requires a sort of analysis that has not yet been approached by modern biochemistry.

The ER membrane can serve as a fundamentally distinct compartment for protein synthesis, with ribosomes, mRNAs, and tRNA synthetases all tethered by their own independent means (Chen et al., 2011; Dang et al., 1983). This subcellular localization may serve as a precise means of translational control – another instance of an emerging understanding that biochemical processes within the cell are carefully

organized even when not separated by membranes (Campanella et al., 2005). I envision a system where the localization of each component of the translational apparatus can be recruited or removed from the ER as a cell grows, divides, differentiates, and responds to external stimuli. Indeed, cells differ widely in their ER enrichment of ribosomes, from professional secretory cells where virtually all ribosomes are ER-bound to leucocytes where the ER is barely detectable (Palade, 1956). I propose that the localization of mRNAs, ribosomes, and associated factors to the ER comprises a dynamic system that can be a critical aspect of translational regulation.

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